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ESTADO PROTROMBÓTICO, FACTORES DE RIESGO CARDIOVASCULAR Y ACTIVIDAD CLÍNICA EN PACIENTES CON LUPUS ERITEMATOSO SISTÉMICO

ELENA MONZÓN MANZANO

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ESTADO PROTROMBÓTICO, FACTORES DE RIESGO CARDIOVASCULAR Y ACTIVIDAD CLÍNICA EN PACIENTES CON LUPUS ERITEMATOSO SISTÉMICO.

Tesis presentada por Mª Elena Monzón Manzano para optar al grado de Doctor por la Universidad Autónoma de Madrid.

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CERTIFICAN

Que Mª ELENA MONZÓN MANZANO ha realizado bajo su dirección el trabajo: "***Estado protrombótico, factores de riesgo cardiovascular y actividad clínica en pacientes con Lupus Eritematoso Sistémico.***".

Este trabajo reúne el interés y condiciones suficientes para considerarlo apto para su presentación como tesis doctoral en el Departamento de Medicina de la Universidad Autónoma de Madrid.

Y para que conste a los efectos oportunos, firmamos el presente escrito en Madrid a 18 de enero de 2021.

_____ _____

Fdo.: Dra. Nora Butta Coll Fdo.: Dr. Francisco Javier López Longo

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ABREVIATURAS

Abreviaturas

ACR: Colegio Americano de Reumatología

ADP: Adenosín difosfato

AINEs: Antiinflamatorios no esteroideos

AL: Anticoagulante lúpico

Anti-ANA: Anticuerpos antinucleares

Anti-cL: Anticuerpos anticardiolipina

Anti-dsDNA: Anticuerpos anti-ADN de doble cadena

Anti-FLs: Anticuerpos antifosfolípidos

Anti-nRNP: Anti-ribonucleoproteínas

Apaf-1: Factor apoptótico 1 activador de proteasas

AT: Antitrombina

Ax: Amplitud a tiempo x

BAFF: Factor activador de células B

BCMA: Antígeno de maduración de células B

BCR: Receptor de células B

BILAG: Escala del grupo de evaluación de lupus de las Islas Británicas

BLyS: Estimulador de linfocitos B

Ca^{2+}: Iones de calcio

CAT: Trombinografía automática calibrada

CD40L: CD40 ligando

CD: Clúster de diferenciación

CDs: Célula dendríticas

CDm: Célula dendrítica mieloide

CDp: Célula dendrítrica plasmacitoide

cfDNA: DNA libre

CFT: Tiempo de formación del coágulo

CMF: Citometría de flujo

CMH: Complejo mayor de histocompatibilidad

CPA: Células presentadoras de antígeno

CPK: Creatín fosfolipasa

CT: Tiempo de coagulación

CTI: Inhibidor de la tripsina del maíz

DAMP: Patrones moleculares asociados a daño

DHEA: Dehidroepiandrosterona

DHEA-s: Dehidroepiandrosterona sulfato

DISC: Complejo de señalización de muerte

ECLAM: Consenso europeo de medida de la actividad de lupus

EDHF: Factor hiperpolarizante

EDRF: Factor de relajación derivado del endotelio

ETP: Potencial endógeno de trombina

EULAR: Liga Europea Contra el Reumatismo

F: Factor

Fadd: Proteína con dominio de muerte

FasL: Ligando Fas

FasR: Receptor de Fas

FITC: Isotiocianato de fluoresceína

FT: Factor tisular

FvW: Factor de von Willebrand

FVIIa: Factor VII activado

GLADEL: Grupo latinoamericano de estudio del lupus

GP: Glicoproteína

HMWH: Quininógeno de alto peso molecular

HSC: Célula madre hematopoyética

IAPs: Proteínas inhibidoras de la apoptosis

ICs: Inmunocomplejos

IFN: Interferón

IL: Interleuquina

ITIM: Dominio de inhibición del inmunorreceptor asociado a tirosina

IV: Índice de velocidad

K: Calicreína

LAI: Índice de actividad del lupus

LDNs: Neutrófilos de baja densidad

LE: Lupus eritematoso

LES: Lupus eritematoso sistémico

LiX: Porcentaje x de coágulo lisado

LT: Tiempo de latencia.

LUMINA: *Lupus in Minorities*

mAb: Anticuerpo monoclonal

MCF: Máxima fortaleza del coágulo

miARNs/miR: Micro-ARN

MK: Megacariocito

MPs: Micropartículas

mTOR: Diana de rapamicina en células de mamífero

NE: Elastasa de neutrófilo

NETs: Rrampas extracelulares de neutrófilo

NKs: Células natural killer

NMDA: N-metil-D-aspartato

PAD-4: Peptidil arginina desaminasa tipo 4

PAF: Factor activador plaquetario

PAI-1/-2: Inhibidor del activador de plasminógeno-1/-2

PCR: Proteína C reactiva

PE: Ficoeritrina

PF4: Factor-4 plaquetario

PGA: Evaluación global del médico

PK: Precalicreína

PMA: Forbol 12-miristato 13-acetato

PMAP: Patrones moleculares asociados a patógenos (PMAP)

PPP: Plasma pobre en plaquetas

PRP: Plasma rico en plaquetas

PS: Fosfatidilserina

PSGL-1: Ligando glicoproteico-1 de la P-selectina

RNAm: ARN mensajero

ROTEM®: Tromboelastometría rotacional

RRP: Receptores de reconocimiento de patrones

scRNA: RNA de cadena simple

sE-selectina: E-selectina soluble

SCCS: Sistema canalicular conectado a la superficie

SIS: Índice del LES del Instituto Nacional de Salud

SLAM: Medida de actividad lupus sistémico

SLICC: Clínicos internacionales colaboradores de lupus sistémico

SNC: Sistema nervioso central

TA: Temperatura ambiental

TAFI: Inhibidor de la fibrinolisis activado por la trombina

TCR: Receptor de células T

TEG: Tromboelastografía

TFPI: Inhibidor de la vía del factor tisular

TL: Tiempo de latencia

TLR: Receptor tipo Toll

TNF: Factor de necrosis tumoral

tPA: Activador tisular del plasminógeno

Tradd: Receptor de TNF asociado a dominio de muerte

TRAP: Péptido agonista del receptor de trombina

TxA_2: Tromboxano A2

uPA: Activador del plasminógeno tipo uroquinasa

UV: Ultravioleta

VDR: Receptor de la vitamina D3

VEB: Virus Epstein-Barr

VSG: Velocidad de sedimentación globular

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RESUMEN

ANTECEDENTES

El lupus eritematoso sistémico (LES) es una enfermedad autoinmune de origen desconocido y patogenia incierta, con una gran variedad de manifestaciones clínicas, por lo que se considera una de las patologías más representativas del grupo de enfermedades sistémicas autoinmunes. Esta enfermedad afecta principalmente a mujeres, especialmente a aquellas en edad fértil.

Esta patología se caracteriza por la producción de autoanticuerpos dirigidos contra numerosos antígenos de las propias células, lo que desencadena diversas manifestaciones cutáneas y sistémicas, que incluso pueden llegar a comprometer la vida del paciente.

En el LES se ha observado un patrón bimodal de mortalidad, en el que las muertes que se producen en la fase temprana de la enfermedad son producidas, mayoritariamente, por infecciones o por la propia actividad del lupus mientras que la fase tardía se caracteriza, fundamentalmente, por complicaciones de origen cardiovascular.

En mujeres que sufren esta enfermedad, se ha observado que la incidencia de enfermedad cardiovascular es de 5 a 9 veces mayor en comparación a mujeres sanas y que, en mujeres jóvenes con LES, el riesgo de sufrir un infarto de miocardio es de hasta 50 veces mayor en comparación con la población sana. Cerca del 20-30% de las muertes que ocurren en estos pacientes se deben al desarrollo de la enfermedad cardiovascular.

Aunque se ha avanzado en el conocimiento del LES y del deterioro de la salud cardiovascular en estos pacientes, aún se desconocen qué factores de riesgo no tradicionales asociados a la enfermedad pueden estar involucrados en este proceso. En el lupus existe una desregulación del sistema inmune y se ha observado que la actividad de la enfermedad se correlaciona con el grado de afectación cardiovascular, lo que sugiere que la enfermedad coronaria se ve agravada por la exposición a un sistema inmune desregulado.

Un alto porcentaje de los pacientes con LES presentan anticuerpos antifosfolípidos (anti-FLs) y estos anticuerpos serían los responsables de la activación basal de las plaquetas y del daño endotelial descrito en estos pacientes. Sin embargo, los pacientes con LES sin anticuerpos anti-FLs también podrían tener algún mecanismo en el plasma o en las plaquetas que contribuyeran a la hemostasia y al estado protrombótico observado en estos pacientes.

HIPÓTESIS

Los pacientes con LES sin anticuerpos anti-FLs presentan un estado protrombótico relacionado con la actividad clínica e inmunológica de la enfermedad que podrían ocasionar daño endotelial y una activación plaquetaria basal.

Además, el estudio del estado protrombótico de estos pacientes utilizando test globales de la coagulación, como la tromboelastometría (ROTEM®) y la trombinografía automática calibrada (CAT) podría aportar nuevos datos sobre los aspectos fisiopatológicos de la enfermedad y ser útiles en el seguimiento de la evolución de estos pacientes.

OBJETIVOS

El principal objetivo es identificar las piezas claves en el estado procoagulante de los pacientes con LES no mediado por anticuerpos anti-FLs, así como evaluar la potencial utilidad del ROTEM® para la caracterización del perfil protrombótico en estos pacientes.

MATERIALES Y MÉTODOS

En el estudio participaron 32 pacientes de sexo femenino diagnosticadas de LES según los criterios definidos por el American Collegue of Rheumatology (ACR), desde enero 2016 hasta enero 2020 en el Servicio de Reumatología del Hospital General Universitario Gregorio Marañón y en el Servicio de Medicina Interna del Hospital Universitario La Paz; sin anticuerpos anti-FLs y sin historial de eventos trombóticos. Ochenta y ocho sujetos sanos fueron reclutados en el Servicio de Donantes del Hospital Universitario La Paz para incluirlos en el grupo control.

Se empleó la citometría de flujo (CMF) para evaluar la función plaquetaria, la exposición de fosfatidilserina (PS) en la superficie plaquetaria, la actividad de las caspasas -3/7, -8, -9 y la formación de agregados entre las plaquetas y los leucocitos.

El ROTEM® fue utilizado para el estudio de la hemostasia global en estos pacientes. Por otro lado, mediante la trombinografía automática calibrada (CAT) se evaluó la capacidad procoagulante asociada a micropartículas (MPs) y la generación de trombina asociada a la producción de trampas extracelulares de neutrófilos (NETs).

Los niveles plasmáticos de PAI-1, E-selectina y LDL-ox se determinaron mediante la técnica ELISA. Los niveles plasmáticos de DNA libre (cfDNA) se determinaron mediante el kit comercial PicoGreen.

La observación de la generación de NETs se realizó mediante microscopía de fluorescencia.

Para el análisis estadístico se utilizó el programa GraphPad-Prism v5.03.

RESULTADOS

El ROTEM® mostró un perfil procoagulante en pacientes con LES. Los niveles plasmáticos de PAI-1, LDL-ox, E-selectina y cfDNA estaban aumentados en pacientes con LES. Además, el grupo de pacientes mostró una activación basal plaquetaria, así como mayor formación de agregados entre las plaquetas y los leucocitos y mayor exposición de PS en la superficie de las plaquetas, no asociado al proceso apoptótico. El CAT mostró un aumento de MPs ricas en factor tisular (FT) y, por último, la generación de trombina asociada a las NETs fue dependiente de la fase de contacto de la coagulación.

CONCLUSIONES

El ROTEM® detectó un estado hipercoagulable en pacientes con LES que parece estar relacionado con el daño endotelial, la existencia de una activación plaquetaria basal, y la duración de la enfermedad. Además, los neutrófilos de los pacientes con LES parecen más propensos a formar más NETs que los neutrófilos de los controles sanos.

Los test globales de la coagulación resultaron útiles para detectar el estado procoagulante en los pacientes con LES sin anticuerpos anti-FLs y sin historia previa ni presencia de trombosis.

1. INTRODUCCIÓN

1.1 ASPECTOS GENERALES DEL LUPUS ERITEMATOSO SISTÉMICO

1.1.1 RESEÑA HISTÓRICA DEL LUPUS ERITEMATOSO SISTÉMICO

El término lupus proviene del latín y significa “lobo”. Aunque se desconoce cómo surgió esta denominación, parece que el origen de la descripción del lupus se remonta a la reseña del herpes estiómenos que Hipócrates había hecho en el 400 a.C. En las descripciones de los siglos XV y XVI, el término lupus se utilizó para aquellas alteraciones destructoras de tejidos semejantes a mordeduras de lobo, que se extienden por la superficie y siguen un curso crónico [1, 2].

En 1833, Laurent Théodore Biett distinguió las ulceraciones faciales, progresivas y destructivas, de otras muy semejantes (lupus tuberculoso), creando así el término *érythème centrifuge,* y limitando su uso a los eritemas faciales con apariencia de enfermedad aguda y con curso crónico, lo que corresponde a lo que actualmente se conoce como lupus discoide [3].

Casi 20 años después, Pierre Cazenave y Ferdinand von Hebra participaron de manera importante en la descripción del lupus. Cazenave designó definitivamente al eritema centrífugo descrito por Biett como lupus eritematoso (LE), siendo más prevalente en el sexo femenino y describiendo que la enfermedad es más común en adolescentes y adultos. En 1846, von Hebra utilizó por primera vez el término “alas de mariposa” para referirse al eritema malar y diez años después publicó en el *Atlas de las enfermedades de la piel* las primeras ilustraciones del LE (Fig. 1).

Figura 1. Dibujo del lupus. Ref.: [Lupus erythematosus. Atlas der Hautkrankheiten. Farblithographie von Anton Elfinger. Heft I, Tafel 6, 59 × 45 cm (1856)].

En 1872, Kaposi describió la forma aguda del lupus, así como ciertas manifestaciones concomitantes que acompañan a la afectación de la piel (pérdida de peso, anemia, artritis, fiebre, etc.) y que incluso podían llegar a afectar a múltiples órganos. También distinguió dos tipos de LE: la forma discoide y la forma diseminada o agregada de la enfermedad. También observó que el LE era más frecuente en mujeres.

Entre 1895 y 1903, William Osler, en Baltimore, y Josef Jodassohn, en Viena, mencionaron la posibilidad de sufrir lupus sin manifestaciones cutáneas y describieron la mayor parte de las complicaciones viscerales de la enfermedad, acuñando de este modo el término *lupus eritematoso sistémico* (LES). Jadassohn realizó la primera revisión del lupus, con más de 400 referencias, donde describió la forma sistémica y la forma discoide, además de incluir la información que se conocía hasta el momento sobre su patología, diagnóstico, pronóstico y tratamiento, contribuyendo de manera importante al entendimiento de la enfermedad [4].

En 1902, Jonathon Hutchinson describió la naturaleza fotosensible de las lesiones cutáneas [5], al mismo tiempo que Sequira y Balean describieron el fenómeno de Raynaud y el lupus nefrítico [6]. En 1923, Emanuel Libman y Benjamin Sacks describieron la endocarditis asociada al lupus, una nueva manifestación de la enfermedad no descrita hasta entonces.

Aunque el diagnóstico del LES mejoró con el paso del tiempo, el entendimiento de la enfermedad siempre limitó su tratamiento. En 1894, Payne usó por primera vez la quinina para el tratamiento del LES. Una década después, Philip S Hench comenzó a usar hormona adrenocorticotropa y cortisona para tratar a estos pacientes. Sulzberger y Witten descubrieron la hidrocortisona, que demostró ser efectiva en el tratamiento del lupus discoide.

En 1938 se usaron sulfonamidas, pero más tarde se descubrió que éstas podían inducir LE. En 1951 se usó por primera vez un fármaco antimalárico para tratar a estos pacientes y solo un año después se usaría por primera vez un inmunosupresor, conocido como mostaza nitrogenada [5]. Actualmente, la inmunomodulación es una aproximación terapéutica muy importante en el tratamiento de la enfermedad.

En 1941, Paul Klemperer acuñó el término *"desórdenes del colágeno vascular"* para describir lo que actualmente se conoce como enfermedades autoinmunes. Con esto se inició la clasificación de este tipo de desórdenes, donde se incluye al LES.

Uno de los pasos más importantes de la investigación biológica de la enfermedad se produjo en 1948, cuando Hargraves, Richmond y Morton describieron las células LE a partir de una punción esternal de pacientes con lupus, demostrando que eran el resultado de la fagocitosis de material nuclear libre, dando lugar a vacuolas con un contenido parcialmente lisado y digerido. En 1950, Haserick y Lewis, al estudiar el mecanismo implicado en la formación de la célula LE, describieron el factor LE en el plasma de estos pacientes al cultivar suero de éstos con médula ósea de controles sanos, resultando ser una inmunoglobulina contra estas células [7].

En el aspecto inmunológico, en 1957, George Friou identificó el factor antinuclear, lo que actualmente se conoce como anticuerpos antinucleares (anti-ANA) [8, 9]. En 1963, Deicher, Colman y Kunkel describieron los anticuerpos anti-DNA. Estas observaciones demostraron un claro proceso autoinmune subyacente a la enfermedad. Más tarde se describirían los anticuerpos anti-ribonucleoproteínas (anti-nRNP), anti-Smith, anti-Ro, Anti-La y los anticardiolipina (anti-cL) [6]. Estos descubrimientos fueron claves para comprender aún más la patogenia del lupus y de otras enfermedades autoinmunes.

1.1.2 EL LES EN LA ACTUALIDAD

Actualmente, el LES es una enfermedad autoinmune, crónica e inflamatoria de origen desconocido, que afecta principalmente a mujeres en edad reproductiva. Se caracteriza por una desregulación del sistema inmune a todos los niveles, donde destaca la presencia de distintos autoanticuerpos contra diferentes partes del organismo, dando lugar a multitud de manifestaciones clínicas que pueden llegar a comprometer la vida del paciente. El curso de la enfermedad en los distintos pacientes tiene gran variabilidad, pero se caracteriza por periodos alternos de brote y remisión, incluso pudiendo padecer brotes crónicos y sufrir los síntomas de forma continua (Fig. 2).

Urowitz *y cols.*, describieron un patrón bimodal de mortalidad en pacientes con LES, donde las infecciones y la propia actividad de la enfermedad son las principales causas de muerte en los primeros cinco años, mientras que, en su fase tardía, hay un repunte de la mortalidad debido a complicaciones cardiovasculares y a fallo orgánico terminal [10].

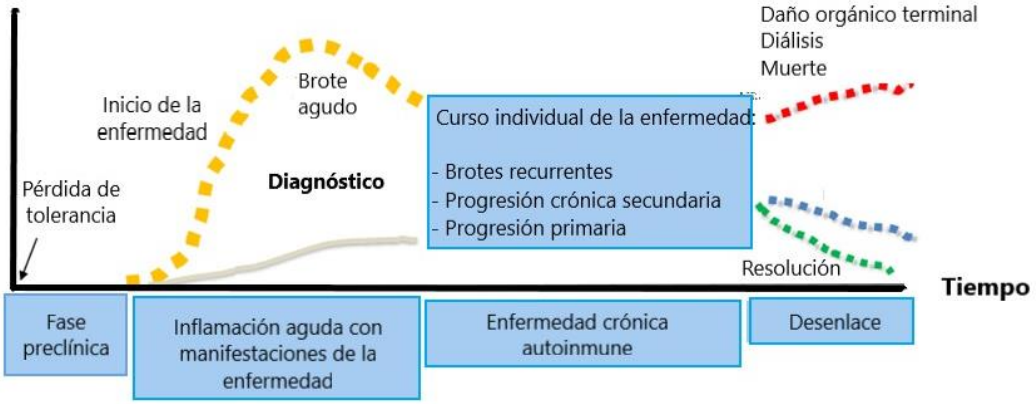


Figura 2. Escenarios posibles de la activación inmune en el curso del LES. Tras la fase preclínica en la que tiene lugar la pérdida de tolerancia, los diferentes desafíos inmunes llevan a manifestaciones agudas del LES, con la posibilidad de tomar diferentes rutas. La activación inmune puede llevar a la cronicidad o a un brote de la enfermedad. Por último, se puede alcanzar la remisión del brote o, si no se controla, puede derivar en un daño orgánico o incluso en la muerte. Adaptada de Rose T, Dörner T. *Drivers of the immunopathogenesis in systemic lupus erythematosus*. Best Pract Res Clin Rheumatol. 2017;31(3):321-333.

1.1.3 DIAGNÓSTICO DEL LES

Criterios de clasificación

La ausencia de pruebas de laboratorio específicas para el diagnóstico del LES y la gran heterogeneidad clínica y serológica de esta enfermedad incentivaron la elaboración de una serie de criterios para facilitar su diagnóstico.

El criterio de clasificación más ampliamente usado para LES es el propuesto por el Colegio Americano de Reumatología (ACR, por sus siglas en inglés). La primera versión fue publicada en 1971 [11] y su objetivo fue distinguir el LES del resto de enfermedades reumáticas. Estos criterios se construyeron a partir de la observación de 245 pacientes que presentaban la enfermedad, procedentes de EEUU y Canadá, según los 52 reumatólogos que participaban en el estudio. Los criterios resultantes fueron 14 y, se debía cumplir al menos 4 criterios de forma seriada o simultánea para diagnosticar al paciente con LES. Estos criterios de clasificación fueron posteriormente revisados en 1982 [12] y en 1997 [13].

En la última revisión de 1997 se eliminaba el test de las células LE y se incluía la presencia de anticuerpos antifosfolípidos (anti-FLs) respecto a las versiones anteriores. Esta versión consta de 11 criterios para diagnosticar la enfermedad.

Petri *y cols.* analizaron las limitaciones del ACR-97 [14]: A) Cuenta con 4 criterios cutáneos, por lo que las manifestaciones mucocutáneas están sobrerrepresentadas. B) ACR-97 omite la hipocomplementemia, que es una de las características más importantes de LES. C) No está validado por un grupo independiente o generalizado de grupos étnicos y D) se deberían incluir criterios no reumatológicos tales como dermatológicos o nefríticos. Así pues, la versión de 1997 tiene una sensibilidad del 86% y una especificidad del 93%, para pacientes con la enfermedad bien establecida, pero la sensibilidad de estos criterios para los pacientes de reciente diagnóstico puede ser significativamente menor, ya que los criterios ACR requieren la presencia de cuatro o más manifestaciones para el diagnóstico de la enfermedad [14].

Para suplir las deficiencias y limitaciones de los criterios ACR, en el 2012 se propuso el Systemic Lupus International Collaborating Clinics Classification Criteria (SLICC), basado en un estudio realizado en 702 pacientes diagnosticados de LES por diferentes expertos. De esta forma surgieron 17 criterios tanto de aspecto clínico como inmunológico. En este caso, para que un paciente sea diagnosticado de LES, tiene que cumplir 4 criterios de los 17 registrados, de los cuales al menos uno tiene que ser un criterio clínico y otro inmunológico, ya sea simultáneamente o de forma seriada. Durante su fase de construcción y respecto al ACR 97, el SLICC 2012 aumentó la sensibilidad de estos criterios (94% vs 86%) y ambos presentaron una especificidad muy semejante (92 vs 93%) [15].

Actualmente, tanto el ACR como el SLICC suelen aplicarse de manera simultánea para un correcto diagnóstico del LES. La tabla 1 presenta los criterios de las distintas versiones del ACR y del SLICC.

	1971 ACR	1982 ACR	1997 ACR	2012 SLICC
	6 puntos	**4 puntos**	**4 puntos**	**4 puntos**
Manifestaciones cutáneas	-Eritema facial. -Rash discoide. -Fenómeno de Raynaud. -Alopecia. -Fotosensibilidad -Ulceras orales o nasofaríngeas.	-Rash malar. -Lesiones de lupus discoide. -Fotosensibilidad -Úlceras orales.	-Rash malar. -Rash discoide. -Fotosensibilidad -Úlceras orales.	-Lupus cutáneo agudo y lupus subcutáneo. -Lupus cutáneo crónico. -Úlceras orales -Alopecia sin cicatrices.

	1971 ACR	1982 ACR	1886 ACR	2012 SLICC
Articulares	**1 punto** -Artritis sin deformidad ≥ una articulación periférica, caracterizado por dolor, sensibilidad o inflamación.	**1 punto** -Artritis no erosiva ≥2 articulaciones periféricas, caracterizadas por dolor, sensibilidad o inflamación.	**1 punto** -Artritis no erosiva ≥2 articulaciones periféricas, caracterizadas por dolor, sensibilidad o inflamación.	**1 punto** -Sinovitis ≥ 2 articulaciones periféricas, caracterizadas por dolor, sensibilidad, inflamación o ≥30 min de rigidez matinal.
Serositis	**1 punto** -Serositis (cualquiera de los siguientes): pleuritis, frote, historia, evidencia tanto de engrosamiento como derrame pleural.	**1 punto** -Serositis (cualquiera de los siguientes): pleuritis, pericarditis.	**1 punto** -Serositis (cualquiera de los siguientes): pleuritis, frote, evidencia de efusión pleural, pericarditis, electrocardiograma anormal.	**1 punto** -Serositis (cualquiera de los siguientes): pleuritis, pleuresía típica >1 día, historia, evidencia de efusión pleural, pericarditis, dolor pericárdico típico >1 día, evidencia electrocardiográfica de fusión pericárdica.
Enfermedad renal	**2 puntos** -Proteinuria ≥3,5 g/día. -Reparto celular.	**1 punto** -Enfermedad renal (cualquiera de los siguientes): proteinuria >0,5 g/día, reparto celular.	**1 punto** -Enfermedad renal (cualquiera de los siguientes): proteinuria >0,5 g/día, reparto celular.	**1 punto** -Enfermedad renal (cualquiera de los siguientes): ratio proteína/creatinina o concentración de proteína en orina de 0,5 g/24 h. Reparto de glóbulos rojos.

	1971 ACR	1982 ACR	1886 ACR	2012 SLICC
Afectación neurológica	**1 punto** -Convulsiones y psicosis en ausencia de tratamientos farmacológicos o alteraciones metabólicas conocidas.	**1 punto** -Convulsiones y psicosis en ausencia de tratamientos farmacológicos o alteraciones metabólicas conocidas.	**1 punto** -Convulsiones y psicosis en ausencia de tratamientos farmacológicos o alteraciones metabólicas conocidas.	**1 punto** -Convulsiones y psicosis en ausencia de tratamientos farmacológicos o alteraciones metabólicas conocidas.
Enfermedad hematológica	**1 punto** -Enfermedad hematológica (Cualquiera de los siguientes): anemia hemolítica, leucopenia (<4000/mm^3) ≥2 ocasiones, trombocitopenia (<10^5/mm^3).	**1 punto** -Enfermedad hematológica (Cualquiera de los siguientes): anemia hemolítica, leucopenia (<4000/mm^3), trombocitopenia (<10^5/mm^3).	**1 punto** -Enfermedad hematológica (cualquiera de los siguientes: anemia hemolítica con aumento de reticulocitos, leucopenia <4000/mm^3 en ≥2 ocasiones, linfopenia <1500 /mm^3 o ≥2 ocasiones trombocitopenia <10^5/mm^3.	**3 puntos** -Anemia hemolítica -Leucopenia o linfopenia (<4000/mm^3, <1000/mm^3 por separado al menos una vez) -Trombocitopenia (<10^5/mm^3) al menos una vez.
Anormalidades inmunológicas	**2 puntos** -Células LE. -Test serológico falso-positivo para sífilis.	**2 puntos** Preparaciones positivas de lupus eritematoso, anti-dsDNA y anti-Sm y test serológico de sífilis falso-positivo. - Anti-ANA positivo.	**1 punto** - Anti-dsDNA, anti-Sm o anti-FLs positivos. - Anti-ANA positivos (por inmunofluorescencia o por ensayo equivalente).	**6 puntos** - Anti-ANA positivo. - Anti-dsDNA positivo (excepto ELISA) en ≥2 ocasiones. - Anti-Sm o anti-FLs -Bajo complemento (C3, C4, o CH50). -Coombs directo en ausencia de anemia hemolítica.

	1971 ACR	1982 ACR	1886 ACR	2012 SLICC
Diagnóstico	Cumplir 4 o más puntos.	Cumplir 4 o más puntos.	Cumplir 4 o más puntos.	Cumplir 4 puntos pero, al menos uno tiene que ser clínico y otro inmunológico.

Tabla 1. Criterios ACR-71, 82, 97 y SLICC 2012. Adaptado de: Diagnostic criteria for systemic lupus erythematosus: A critical review.

En 2019 se han publicado nuevos criterios de clasificación para el LES por el ACR/EULAR (European League Against Rheumatism, por sus siglas en inglés) [16]. Estos nuevos criterios tienen una sensibilidad del 96.1% y una especificidad del 93.4%, valores superiores a los demostrados por el ACR-97 y el SLICC-2012, ya mencionados anteriormente. Todos los pacientes que sean diagnosticados con LES deben presentar un título de anti-ANA ≥ 1:80 en células epiteliales HEp-2 o un test que resulte positivo y que sea equivalente a éste. Si el paciente no cumple estas condiciones, no puede ser diagnosticado como paciente con LES y, por el contrario, en aquellos pacientes que lo cumplan, además se deberán aplicar los criterios descritos en la tabla 2.

Como norma, se establece que los criterios que tengan alguna explicación más allá del LES no deberán tenerse en cuenta. Por el contrario, si realmente se asocian a la enfermedad, tendrán que tenerse en cuenta aunque solo hayan tenido lugar una única vez, y los criterios no tienen por qué solaparse en el tiempo, aunque uno de ellos sí debe ser un criterio clínico y el resultado final debe ser ≥ 10 puntos. Por último, dentro de cada dominio, solamente se tendrá en cuenta aquel criterio con la puntación más alta.

Dominio clínico	Puntos	Dominio inmunológico	Puntos
Constitucional Fiebre	2	**Anticuerpos antifosfolípidos** Anti-cL o anti-β2GP1 o AL	2
Hematológico Leucopenia Trombocitopenia Hemólisis autoinmune	 3 4 4	**P** **oteínas del complemento** Bajo C3 o bajo C4 Bajo C3 y bajo C4	 3 4
Neuropsiquiátrico Delirios Psicosis Convulsiones	 2 3 5	**Anticuerpos específicos de LES** Anti-dsDNA* o anti-Smith	6
Mucocutáneo Alopecia no cicatrizal Úlceras orales Cutáneo subagudo o lupus discoide Lupus cutáneo agudo	2 2 4 6	–	–
Serológico Efusión pleural o pericárdica Pericarditis aguda	5 6	–	–
Musculoesquelético Afectación articular	 6	–	–
Renal Proteinuria >0,5 g/24 h Clase II o V de nefritis lúpica Clase III o IV de nefritis lúpica	4 8 10	–	–

Tabla 2. Criterios ACR/EULAR 2019. Adaptado de *Aringer M y cols. 2019 European League Against Rheumatism/American College of Rheumatology Classification Criteria for Systemic Lupus Erythematosus. Arthritis Rheumatol. 2019 Sep;71(9):1400-1412.*

Índices de actividad

Evaluar la actividad de la enfermedad es básico para tomar decisiones terapéuticas frente a estos pacientes. Los criterios de clasificación del LES no son suficientes para describir el grado de la actividad de la enfermedad y, por ello, se han desarrollado y validado varios índices de actividad de la enfermedad [17].

Estos índices definen el grado de actividad de la enfermedad en cualquier momento, pudiendo oscilar desde la remisión de la enfermedad o actividad mínima hasta la actividad grave que puede comprometer incluso la vida del paciente [18, 19]. Dichos índices pueden medir la actividad de forma global o específica, para cada órgano o sistema (Tabla 3).

Índices con medidas globales de la actividad	Índices específicos para cada órgano o sistema
European Consensus Lupus Activity Measure (ECLAM): permite medir la actividad de la enfermedad de forma retrospectiva e incluye la velocidad de sedimentación globular (VSG) y los niveles del complemento. *Lupus Activity Index (LAI):* Combina la evaluación de 4 sistemas y 3 valores de laboratorio. Más simple que SLEDAI. *National Institute of Health SLE Index SCORE (SIS):* incluye 17 items, pero es subjetiva, ya que valora fatigas, artralgias y mialgias. *Systemic Lupus Activity Measure (SLAM):* incluye factores subjetivos, como la artralgia y la fatiga, por lo que es menos fiable. *SLE Disease Activity Index (SLEDAI):* índice más ampliamente usado en la práctica clínica habitual.	*British Isles Lupus Assessment Group Scale (BILAG):* evalúa las manifestaciones clínicas específicas de distintos órganos, así como la aparición de nuevos brotes de la enfermedad.

Tabla 3. Índices de medición de la actividad del LES.

Estos índices se han establecido a través de estudios observacionales y han mostrado ser buenos predictores del daño y de la mortalidad, teniendo la capacidad de reflejar cambios en la actividad de la enfermedad. Además han demostrado buena correlación entre ellos [20].

Escala SLEDAI

La escala SLEDAI es un índice de actividad lúpica, publicada en 1992, que recoge manifestaciones de 9 órganos o sistemas y que evalúa la actividad de la enfermedad en los últimos 10 días [21]. Es el más utilizado en la práctica diaria e incluye una revisión por diversos sistemas orgánicos (sistema nervioso, cardiovascular, esquelético…) del paciente. Consiste en una lista de 24 manifestaciones en la que cada una de ellas tiene una puntuación determinada y donde las manifestaciones más serias (neurológicas, renales y vasculitis) reciben una mayor puntuación [22]. La puntuación total de este índice oscila entre 0 y 105 y, según el resultado, se clasificará al paciente en remisión, o con presencia de una actividad leve/moderada o grave (Tabla 4).

Actividad leve o moderada	Actividad grave
Un cambio de más de 3 puntos en SLEDAI	**Un cambio de más de 12 puntos en SLEDAI**
Empeoramiento de lupus discoide o de nueva aparición, lupus ampolloso, fotosensibilidad, vasculitis cutánea profunda, úlceras nasofaríngeas, fiebre, pleuritis, pericarditis o artritis.	Empeoramiento de LES-SNC o de nueva aparición, vasculitis, nefritis, miositis, Pk <60000, anemia (Hb <7% o una disminución de >3%).
Aumento de la prednisona sin superar > 0,5 mg/kg/día.	Prednisona a > 0,5 mg/kg/día.
Añadir antiinflamatorios no esteroideos (AINES) o hidroxicloroquina.	Necesidad de ciclofosfamida, azatioprina, metotrexato u hospitalización.
>/= 1 de incremento en el score de evaluación global del médico (PGA), pero no más de 2,5.	Aumento de PGA > 2,5.

Tabla 4. Características de la actividad leve/moderada y grave del LES.

Existe una versión modificada de este índice, realizada en el año 2002 y conocido como SLEDAI-2K. En esta actualización se incluyen ítems como la alopecia, el rash, úlceras y proteinurias persistentes, sin necesidad de ser de nueva aparición (Tabla 5).

Puntuación	Manifestación	Definición
8	Convulsiones	De inicio reciente. Excluir causas metabólicas, infecciosas y relacionadas con fármacos.
8	Psicosis	Capacidad alterada para la actividad diaria debido a alteración grave en la percepción de la realidad. Incluye alucinaciones, incoherencia, asociaciones ilógicas, contenido mental escaso, pensamiento ilógico, raro, desorganizado y comportamiento catatónico. Excluir la presencia de uremia y fármacos.
8	Síndrome orgánico cerebral	Función mental alterada con falta de orientación, memoria, u otras funciones intelectuales, de comienzo rápido y manifestaciones clínicas fluctuantes. Incluye disminución del nivel de conciencia con una disminución en la capacidad para focalizar, e incapacidad para mantener la atención, y al menos dos de los siguientes: alteración de la percepción, lenguaje incoherente, insomnio o mareo matutino, actividad psicomotora aumentada o disminuida. Excluir causas infecciosas, metabólicas y relacionadas con fármacos.
8	Alteración visual	Retinopatía lúpica. Incluye cuerpos citoides, hemorragias retinianas, exudados serosos o hemorragias en la coroides, neuritis óptica (no debido a hipertensión, infección o fármacos).
8	Alteración en nervios craneales	Neuropatía motora o sensitiva de nuevo comienzo que involucre nervios craneales.
8	Cefalea lúpica	Grave, persistente. Puede ser migrañosa pero no responde a analgésicos narcóticos.
8	Accidente cerebrovascular	De reciente comienzo. Excluir arteriosclerosis.

Puntuación	Manifestación	Definición
8	Vasculitis	Ulceración, gangrena, nódulos dolorosos sensibles, infartos periungueales, hemorragias en astilla. Vasculitis confirmada por biopsia o angiografía.
4	Miositis	Debilidad o dolor proximal asociado a elevación de la creatinfosfoquinasa (CPK)/aldolasa, cambios electromiográficos o miositis confirmada por biopsia.
4	Artritis	Más de dos articulaciones dolorosas y con signos inflamatorios.
4	Cilindros urinarios	Cilindros hemáticos o granulosos.
4	Hematuria	>5 hematíes/campo. Excluir otras causas (litiasis, infección).
4	Proteinuria	> 5 g/24 h. De reciente comienzo o aumento de la proteinuria ya conocida en más de 0,5 g/24 h.
4	Piuria	> 5 leucocitos/campo. Excluir infección.
2	Nuevo exantema	Comienzo reciente o recurrente de un exantema inflamatorio.
2	Alopecia	De comienzo reciente o recurrente.
2	Úlceras bucales	Comienzo reciente o recurrente de úlceras bucales o nasales.
2	Pleuritis	Dolor pleurítico con roce o derrame, o engrosamiento pleural.

Puntuación	Manifestación	Definición
2	Pericarditis	Dolor pericárdico con al menos uno de los siguientes: roce, derrame, cambios electrocardiográficos o confirmación mediante ecocardiografía.
2	Bajo complemento	Disminución de los niveles de CH50, C3, C4 o por debajo del límite inferior del laboratorio.
2	Anti-DNA	> 25%. Técnica de Farr o por encima del valor habitual del laboratorio.
1	Fiebre	> 38ºC. Excluir infección.
1	Trombopenia	< 10^5 plaquetas/mm^3.
1	Leucopenia	< 3000 células/mm^3. Excluir fármacos.

Tabla 5. Índice de actividad según el SLEDAI-2K.

El SLEDAI-2K es fácil de utilizar y cuenta con una serie de ventajas e inconvenientes, que se recogen en el siguiente cuadro (Tabla 6).

Ventajas	Desventajas
- Fácil uso, rápido y ágil. - Aplicable a cualquier edad (aunque en niños se emplea el SLEDAI-IALES). - Revisión amplia por distintos sistemas. - Puede predecir daño y morbilidad, influyendo así en el pronóstico.	- Pocos parámetros hematológicos. - No permite asignar valores distintos para una misma manifestación (misma puntuación para trombocitopenia, independientemente del número de plaquetas). - No menciona manifestaciones cutáneas en profundidad [23, 24].

Tabla 6. Ventajas y desventajas del empleo de la escala SLEDAI-2K.

Además, existen dos modificaciones del índice SLEDAI: el MEX-SLEDAI, diseñado para países en vías de desarrollo, creado por investigadores mexicanos y que excluye parámetros inmunológicos como los niveles del complemento [25], y el SELENA-SLEDAI, diseñado para mujeres y grupos tratados con estrógenos y progesterona [26].

1.1.4 EPIDEMIOLOGÍA

A lo largo de los años, se ha observado un aumento en la prevalencia del LES, posiblemente debido a las mejoras en el conocimiento y diagnóstico de la enfermedad. Aunque existen más de 60 estudios que mencionan la incidencia de la enfermedad [27], en España existen pocos artículos con datos actualizados y consistentes [28].

La incidencia de la enfermedad se distribuye ampliamente a nivel mundial, pues estudios han demostrado que el LES tiene una tendencia racial, siendo más frecuente en individuos no caucásicos. Por ejemplo, en EEUU, la enfermedad es más frecuente en afroamericanos, hispanos y asiáticos en comparación a sujetos caucásicos [27].

La mayor prevalencia e incidencia del LES descrita hasta la fecha se encuentra en Norteamérica, siendo de 23,2/100000 personas al año y de 241/100000 personas, respectivamente. Sin embargo, las incidencias más bajas fueron registradas en África y Ucrania y la prevalencia más baja se encontró en el norte de Australia (0 casos en una muestra de 847 personas) [29].

Respecto a la situación del LES en España, el Proyecto EPISER 2000, cuyo objetivo fue estudiar la prevalencia de distintas enfermedades reumatológicas que afectan a la población española en el año 2000 [30] mostró que la prevalencia del LES en la población española era de 91/100000 habitantes [31].

Más tarde, con el objetivo de dar continuidad al proyecto y actualizar los datos de prevalencia de las enfermedades reumáticas en España, se puso en marcha el Proyecto EPISER 2016 [32, 33]. Los datos de dicho estudio muestran que el LES tiene una prevalencia de 210/100000 habitantes [34], prevalencia superior a lo anteriormente publicado en otros países europeos.

1.1.5 ETIOLOGÍA

Actualmente se piensa que factores genéticos, ambientales, hormonales, agentes infecciosos, la edad, el sexo, la raza y la epigenética pueden estar involucrados en las alteraciones inmunes que tienen lugar en el LES (Fig. 3).

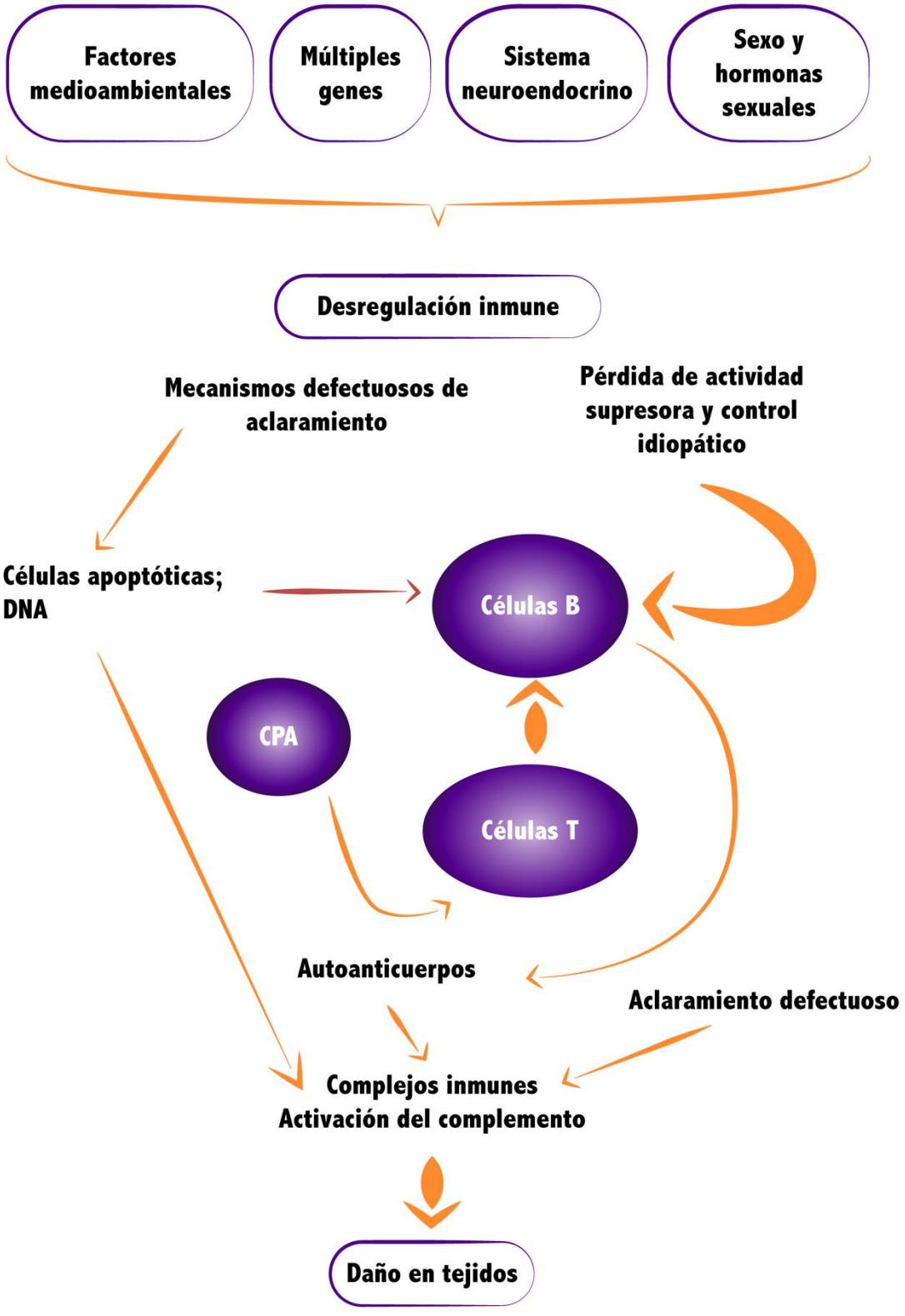


Figura 3. Patogénesis del LES. CPA: células presentadoras de antígenos. Adaptado de C C Mok, C S Lau. *Pathogenesis of systemic lupus erythematosus.* J Clin Pathol 2003;56:481–490.

Factores genéticos

Aparentemente el LES tiene un componente genético, ya que su aparición se ha observado en miembros de una misma familia, con un porcentaje similar en poblaciones europeas, latinoamericanas y afroamericanas (8-10%), así como una alta concordancia entre gemelos monocigóticos (24-50%) con un porcentaje muy superior que el encontrado en gemelos dicigóticos (2-5%) [35]. La fracción de LES atribuible a la carga genética se ha estimado en un 66% [36].

Para conocer los genes involucrados en LES, se han realizado estudios de ligamiento, de asociación y de expresión génica.

Respecto a los estudios de ligamiento, se han identificado más de 70 loci relacionados con los productos de los genes del complemento (C2, C4 y C1q), cuya consecuencia es un aclaramiento parcial del debris celular, lo que favorece la aparición de autoantígenos [37].

Estudios de asociación de genomas (GWAS-*Genome Wide Association Studies*) han mostrado que pacientes con LES suelen presentar mutaciones en genes que codifican proteínas del complejo mayor de histocompatibilidad de clase II (CMH-II), genes que codifican componentes de la vía de señalización del interferón (IFN) de tipo I (IRF5, IRAK, TREX1), genes que participan en la cascada del complemento (ITGAM), así como en genes que codifican proteínas que están implicadas en la regulación del sistema inmune, en la maduración de células, en la inflamación y en la tolerancia inmunológica [38].

Por otro lado, en estudios de expresión génica se ha podido observar que los neutrófilos de estos pacientes tienen mayor expresión del gen ITGAM, que codifica para la cadena α de la integrina αM/β2, la cual está implicada en la activación de leucocitos, adhesión de granulocitos, monocitos y macrófagos, así como en la captura de partículas cubiertas por proteínas del complemento. Además, tener más de dos copias del gen TLR7, también aumenta el riesgo de padecer LES [39, 40].

Agentes infecciosos

La exposición a agentes fúngicos, parasitarios, bacterianos y/o virales pueden causar aberraciones en el funcionamiento del sistema inmune, favoreciendo el desarrollo de la enfermedad [41].

Un gran número de virus y bacterias pueden producir superantígenos que se unen al receptor de células T (TCR) y a las moléculas del CMH-II, activando a los linfocitos T e induciendo distintas reacciones autoinmunes que desencadenan enfermedades de esta naturaleza [42].

Algunos virus también pueden inducir la expresión del gen de IFN-I, conocido como la firma del interferón, el cual está íntimamente relacionado con muchas enfermedades autoinmunes [43].

El virus de Epstein-Barr (VEB) ha sido señalado como un desencadenante potencial de LES, pues se han observado títulos elevados de anti-VEB en estos pacientes, con una prevalencia del 99% de infecciones de este tipo en pacientes jóvenes con LES, en comparación al 70% observado en el grupo control [44].

Factores ambientales

Entre los factores de riesgo ambiental se incluyen, entre otros, la exposición a la luz ultravioleta (UV), a múltiples fármacos, a silicatos, a la suciedad y al hábito de fumar (Fig. 4).

La luz UV produce la apoptosis de los queratinocitos de la piel y el aclaramiento defectuoso característico en estos pacientes puede causar la precipitación de inmunocomplejos (ICs) en la piel, produciendo inflamación. Por ello se piensa que la exposición al sol puede jugar un papel clave en la patogénesis del LES. El 80% de los pacientes con LES muestran fotosensibilidad y deben protegerse cuidadosamente de la radiación solar, lo que suele estar ligado a una deficiencia de vitamina D [45].

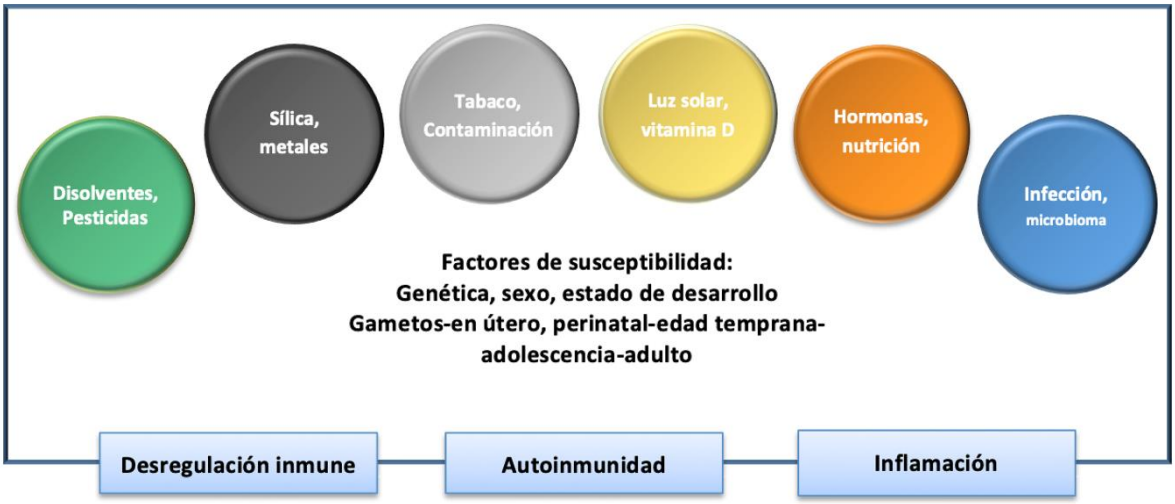


Figura 4. Factores ambientales que influyen en el desarrollo del LES. Además de la importancia de los genes, el sexo y la edad, existen factores ambientales que contribuyen a los cambios epigenéticos y a una desregulación inmune, lo que lleva a la pérdida de tolerancia inmune, a un aumento de la inflamación y a una mayor susceptibilidad a desarrollar LES. Adaptado de CG. Parks. *Understanding the role of environmental factors in the development of systemic lupus erythematosus.* Best Practice & Research Clinical Rheumatology (2017): 1-15

Por otro lado, ciertos fármacos que inhiben la metilación del ADN [46], como la quinidina, procainamida e hidralazina, son capaces de inducir la formación de anti-ANAs, dando lugar a lo que se conoce como lupus farmacológico, siendo las manifestaciones dermatológicas y articulares las que se observan más frecuentemente y que suelen desaparecer pasadas unas semanas tras la retirada del fármaco. En estos casos, no hay generación de anti-DNA de doble cadena (anti-dsDNA), lo que ayuda a diferenciarlo del lupus idiopático.

El tabaco contiene numerosos agentes tóxicos que podrían causar mutaciones genéticas y alterar la respuesta inmune humoral y celular [47] favoreciendo el desarrollo de enfermedades autoinmunes a largo plazo, ya que este hábito puede activar a los macrófagos alveolares, inducir la actividad de la enzima mieloperoxidasa y producir radicales libres [48], aunque existen discordancias en los resultados de distintos estudios sobre el papel del tabaquismo en el aumento del riesgo de sufrir LES.

En ámbitos ocupacionales, residenciales, rurales y en entornos urbanos se ha observado una asociación entre la exposición a la sílica y el incremento de desarrollar LES [49]. La sílica actúa como un adyuvante inmune que induce la producción de interleuquina (IL) tipo 1 y factor de necrosis tumoral (TNF) tipo α, así como la apoptosis y liberación de antígenos intracelulares, incrementa los niveles de citoquinas proinflamatorias, de estrés oxidativo y la respuesta de células T, además de disminuir el número de células T reguladoras. Todo ello produce una desregulación del sistema inmune y favorece el desarrollo del LES [50].

Influencia del género

El LES es más frecuente en mujeres que en hombres, especialmente en aquellas con edad fértil, con un ratio de 8-15:1, mientras que en el lupus juvenil y tardío el ratio es de 2-8:1.

Individuos con el síndrome de Klinefelter, caracterizado por tener un genotipo 47XXY, tienen una probabilidad 14 veces mayor de padecer LES en comparación con hombres XY, lo que sugiere que la carga de un cromosoma X extra es un importante contribuyente a la susceptibilidad de desarrollar LES [51]. Esto se debe al hecho de que en el cromosoma X se sitúan varios genes implicados en la susceptibilidad de sufrir LES, tales como IRAK1 y TLR7 [52]. Aunque hay varios casos registrados, no es frecuente que mujeres con el síndrome de Turner (un cromosoma X ausente o incompleto) sufran LES [53].

Las manifestaciones clínicas del LES son diferentes en hombres y en mujeres, siendo la artritis, la artralgia y la probabilidad de sufrir un brote menos frecuente en hombres [54, 55]. En ellos se han

registrado prevalencias más bajas de anti-Ro [54] y anti-La y una alta prevalencia de anti-Smith, anti-dsDNA e hipocomplementemia [56].

Influencia de la edad

La edad en la que comienzan a aparecer los primeros signos de la enfermedad tiene un importante impacto en las manifestaciones clínicas del lupus [57]. Aunque puede aparecer a cualquier edad, suele ser más tardío en hombres que en mujeres. Se calcula que el rango medio de edad en el que se diagnostica la enfermedad es de 35-45 años y se habla de lupus tardío cuando se diagnostica a partir de los 50 años.

El lupus juvenil representa el 10-20% de todos los casos de LES [58] y la actividad de la enfermedad en niños es más pronunciada en comparación a los adultos [59]. Los ratios de incidencia y prevalencia son considerablemente menores en comparación con los ratios en adultos, siendo la incidencia anual de lupus juvenil (menores de 16 años) de 1 por cada 100000 personas en Europa y Norteamérica [60].

Influencia de factores hormonales

El hecho de que el LES afecte principalmente a mujeres en edad fértil y que se pueda agudizar tras el parto o tras la administración de anticonceptivos indica que factores hormonales están implicados en el desarrollo de la enfermedad.

Hay diferentes hipótesis que se centran en el papel de los estrógenos, progesterona, testosterona, dehidroepiandrosterona (DHEA) y la prolactina en la respuesta del sistema inmune.

Estudios realizados en ratones NZB/NZW, que muestran un síndrome parecido a lupus, castrados y no castrados, muestran que los estrógenos aceleran la aparición del LES. Por ello se piensa que los estrógenos, que han sido relacionados con la estimulación de timocitos, linfocitos B y T, macrófagos, citoquinas y moléculas de adhesión endotelial (VCAM, ICAM), tienen un efecto activador del sistema inmune [61]. Por el contrario, la testosterona tendría propiedades supresoras. Se ha observado que los niveles de DHEA, precursor de la testosterona, están disminuidos, lo que impide una buena regulación del sistema inmune.

La progesterona y la prolactina también intervienen en el LES, ya que la primera es capaz de disminuir la proliferación de linfocitos T y de incrementar el número de células $CD8^+$ [62, 63], mientras que la prolactina participa en la respuesta inmune, habiendo receptores de esta hormona en linfocitos y monocitos humanos y, aunque no se conoce bien el mecanismo por el

cuál la prolactina afecta a la inmunidad, niveles elevados de ésta se han asociado a brotes de la enfermedad. Por el contrario, altos niveles de progesterona y estrógenos promueven la respuesta de las Th2, que favorece la producción de anticuerpos [63].

Por otro lado, las mujeres con LES suelen presentar niveles normales de estradiol y muestran, a su vez, un incremento de 16-α-hidroxiestrona con una actividad estrógena elevada y bajos niveles de testosterona. Otros estudios han demostrado que el uso de hormonas exógenas aumenta el riesgo de sufrir LES [64, 65].

Influencia de la raza/etnia

Existen varios estudios de cohortes, como GLADEL (*Grupo Latino-Americano de Estudio del Lupus*), PROFILE , cohorte multiétnica y multicéntrica y LUMINA (*Lupus in Minorities: Nature vs Nurture*), de EEUU, cuyos objetivos son realizar estudios longitudinales de seguimiento y desenlace de la enfermedad, estudiando el papel que juegan los factores genéticos y no genéticos en diferentes grupos étnicos y durante el transcurso de la enfermedad [66-68].

Problablemente existan manifestaciones clínicas diferentes entre distintas poblaciones, ya que aquellos con descendencia africana o amerindia tienen mayor riesgo de sufrir lupus nefrítico [69], en comparación a los descendientes de europeos [70]. Sin embargo, aunque entre los descendientes europeos destaque la producción de anticuerpos, los del sur suelen tener mayor afectación renal mientras que, en la parte occidental, destaca la presencia de serositis [71]. En general, la actividad de la enfermedad es mayor en no caucásicos que en caucásicos y suelen presentar afectación renal, así como manifestaciones hematológicas, serosíticas, neuropsiquiátricas y anormalidades inmunes [72, 73].

Epigenética

Las modificaciones postraduccionales que implican alteraciones en la expresión génica como metilación del ADN, modificación postraduccional de histonas y microARNs (miARNs o miR) influyen en la aparición del LES.

Se ha observado hipometilación del ADN en células T $CD4^+$ de pacientes con LES y en regiones reguladoras de varios genes implicados en la patogénesis de la enfermedad. Ejemplo de esto son los genes que codifican, entre otros, al CD40 ligando (CD40L), al CD70, a la perforina, a la IL-10 y a la IL-13.

La hipometilación del ADN puede causar el aumento en la producción de citoquinas y de anticuerpos y la hiperactividad de linfocitos T $CD4^+$. La hipometilación de genes en las células T $CD4^+$ de pacientes con LES se correlaciona con la actividad de la enfermedad [74].

Las modificaciones covalentes de histonas pueden alterar la estructura de la cromatina y con ello, regular la expresión génica. Se han observado varias modificaciones en histonas de diferentes tipos celulares en los pacientes con LES.

Los miARNs son ácidos ribonucleicos no codificantes de cadena corta que se unen a secuencias homólogas de los transcritos de mRNA y regulan la expresión génica. Uno de los miARNs que influyen en la enfermedad es el miR-146a, cuya expresión se encuentra disminuida en células T $CD4^+$ de pacientes con LES y se correlaciona negativamente con la actividad de la enfermedad [75].

Alteraciones del sistema inmune

En el LES se ha descrito una serie de alteraciones en los componentes tanto de la inmunidad innata como de la inmunidad adaptativa en estos pacientes. Entre estas alteraciones se incluye la aparición de autoanticuerpos con distinta especificidad, la alteración de la función de las células T, la modificación de la fagocitosis y cambios en oncogenes, entre otros [76].

1. Sistema inmune innato

La inmunidad innata es considerada como la primera línea de defensa ante una infección. Se caracteriza porque no es específica de antígeno, es decir, se trata de una respuesta inespecífica. Además, tampoco tiene memoria inmunológica y constituye una respuesta máxima inmediata.

La respuesta inmune innata tiene la capacidad de diferenciar estructuras microbianas conservadas conocidas como patrones moleculares asociados a patógenos (PMAP) a través de los receptores de reconocimiento de patrones (RRP), activando ciertos mecanismos intramoleculares que orientan la respuesta de la inmunidad adaptativa en LES.

Los componentes de la inmunidad innata que se han relacionado con LES son, entre otros:

- El sistema del complemento.
- Componentes celulares: neutrófilos, macrófagos, natural killer (NK), células dendríticas (CDs), IFNs y receptores tipo TOLL.

a) Sistema del complemento

El sistema del complemento está constituido por más de 30 proteínas y sus funciones principales son: citólisis, opsonización y fagocitosis, la activación de la inflamación y el aclaramiento [77].

En la mayor parte de estos pacientes existe una deficiencia adquirida de los niveles de las proteínas C1q, C3 y C4 [78]. La disminución de estas proteínas tiene un efecto negativo sobre la fagocitosis y el aclaramiento de las células apoptóticas [79], ya que impide el correcto aclaramiento de los ICs, lo que da lugar a la deposición de éstos sobre los tejidos y órganos, produciendo inflamación y daño tisular [80]. Además de ser la primera fuente de autoantígenos, la deposición de los ICs, a su vez, activa el sistema del complemento lo que lleva a un mayor consumo de estas proteínas, explicando así que la disminución de los niveles de C3 y C4 se relacione con la actividad de la enfermedad en estos pacientes [81]. Aunque se desconoce cómo afecta esto a la autoinmunidad, esta deficiencia del sistema del complemento contribuye a la patogenia del LES [82]. Además, los niveles reducidos de estas proteínas serían los responsables de un fallo en la selección negativa de células B, lo que favorecería la supervivencia y propagación de estas células autorreactivas características del LES [83].

b) Monocitos y macrófagos: fagocitosis de células apoptóticas y necróticas

Los monocitos son un tipo de glóbulos blancos que se diferencian a macrófagos una vez que son reclutados en los tejidos. La función principal de los macrófagos es llevar a cabo la fagocitosis.

Bajo circunstancias normales, las células apoptóticas son eficientemente fagocitadas por macrófagos en la fase temprana del proceso sin inducir inflamación ni respuesta inmune [84, 85]. Varios estudios han demostrado que los macrófagos de los pacientes con LES presentan una función fagocítica disminuida en comparación a los controles [86, 87], haciendo que las células apoptóticas sirvan como señal inmunogénica activando a las células T y B autorreactivas para, finalmente, generar autoanticuerpos.

Ya en 1975, Svensson *y cols.* describieron que en la mitad de los pacientes con LES existía una actividad fagocítica alterada [88]. Además, Muñoz observó que las células madre $CD34^+$ hematopoyéticas procedentes de sangre periférica de estos pacientes mostraban una diferenciación a macrófagos reducida [89, 90] y que, además, estos macrófagos tenían disminuida su capacidad fagocítica y de adherencia [91]. También se demostró que los macrófagos de pacientes con LES expresan menor número de receptores de adhesión CD44, implicados en el proceso de aclaramiento [92].

La proteína C1q como ya se ha comentado, junto con la proteína C reactiva (PCR) e IgM son elementos moleculares importantes en la patogénesis del lupus [93]. Estos elementos mejoran el proceso de aclaramiento de células apoptóticas por macrófagos, ya que se unen a los autoantígenos y previenen la respuesta inflamatoria. IgM reconoce epítopos expresados en estas células y, junto con C1q, facilita la fagocitosis de las células muertas por macrófagos, mientras que la PCR se une al material apoptótico, resultando en una amplificación de la vía clásica del complemento. Se ha observado una disminución en los niveles de IgM [94] y de PCR, así como un consumo de las proteínas del complemento en los pacientes con un brote de LES, lo que explicaría la alteración en el aclaramiento de células apoptóticas, pudiendo resultar en una acumulación de células muertas en la sangre periférica de estos pacientes [95].

c) Neutrófilos y NETosis

Los neutrófilos son la primera línea de defensa ante una infección. Además de ser potentes estimuladores de la inmunidad adaptativa, también tienen otras funciones inmunes, como la fagocitosis, la secreción de citoquinas y la producción de agentes antimicrobianos [96].

La NETosis, proceso llevado a cabo por los neutrófilos para la eliminación de microorganismos, es una forma de muerte celular en la cual liberan una estructura conocida como trampas extracelulares de neutrófilos (NETs), consistente en una malla de cromatina descondensada y proteínas, principalmente con carga positiva (Tabla 7) [34, 97-99]. La función principal de estas redes es facilitar la adhesión de patógenos microbianos y virales con carga negativa, de forma que tanto histonas como ácidos nucleicos pueden ejercer una actividad bactericida, protegiendo al organismo de infecciones.

Componente	Localización celular
Elastasa, mieloperoxidasa, catepsina G, lactoferrina, BPI y gelatinasa	Gránulos
DNA, histonas de tipo H1, H2A, H2B, H3 y H4	Núcleo
Actina y miosina-9	Citoesqueleto
Calprotectina	Peroxisoma

Tabla 7. Componentes de las NETs y su localización celular dentro de los neutrófilos.

Aunque en 1996 el japonés Takei describió por primera vez la respuesta de los neutrófilos al tratarlos con el activador f*orbol*-12-*miristato*-13-*acetato* (PMA) [100], no fue hasta el trabajo de Brinkmann *y cols.* en 2004 [34] cuando se comenzó a estudiar la implicación del proceso de NETosis y las NETs en distintas enfermedades, incluyendo el LES.

El proceso de NETosis puede comenzar por una amplia variedad de estímulos tanto orgánicos como inorgánicos [34, 101, 102]. Una vez que los neutrófilos se activan, sufren una serie de cambios morfológicos característicos de este proceso. La NETosis comienza con la descondensación de la cromatina y la pérdida de la forma lobular característica de estas células. Durante el proceso, ciertas enzimas, incluyendo la elastasa de neutrófilo (NE), se liberan de los gránulos de neutrófilos y se translocan al núcleo, donde se unen a las histonas y las degradan, siendo finalmente citrulinadas por la peptidil arginina desaminasa tipo 4 (PAD-4), desmantelando así el nucleosoma. En el siguiente paso, la membrana de los gránulos y del núcleo se degradan, lo que permite que el contenido granular se mezcle con la cromatina descondensada. En el último paso, la membrana celular se rompe y las NETs funcionalmente completas son liberadas a la matriz extracelular [103] (Fig. 5), ocasionando la muerte del neutrófilo (NETosis suicida) [103, 104]. Clark *y cols.* demostraron que, a veces, tras la formación de las NETs, los neutrófilos podían mantener sus funciones (NETosis vital) [105, 106], o, por el contrario, finalizaba con la muerte del neutrófilo (NETosis suicida) [104].

Los neutrófilos de los pacientes con LES han mostrado tener anormalidades tanto a nivel fenotípico como funcional. Los pacientes con LES presentan más neutrófilos apoptóticos en circulación en comparación con controles sanos y su número se correlaciona con la actividad de la enfermedad, pudiendo proporcionar una fuente extra de autoantígenos [107, 108].

En condiciones normales, los macrófagos fagocitan y eliminan eficientemente las NETs, proceso favorecido por C1q y por la DNasa I. La fagocitosis de las NETs es un proceso inmunológico silencioso ya que no induce la secreción de citoquinas proinflamatorias [109]. Se ha observado que los pacientes con LES activo tienen menor capacidad para degradar las NETs debido a una baja actividad de la DNasa, bien por una causa genética [110] o bien debido a la presencia de inhibidores de DNasa I y de anti-DNA, que reconocen un epítopo conservado cerca del sitio catalítico de la DNasa, protegiendo a las NETs de la degradación [111] y participando en el desarrollo de la autoinmunidad.

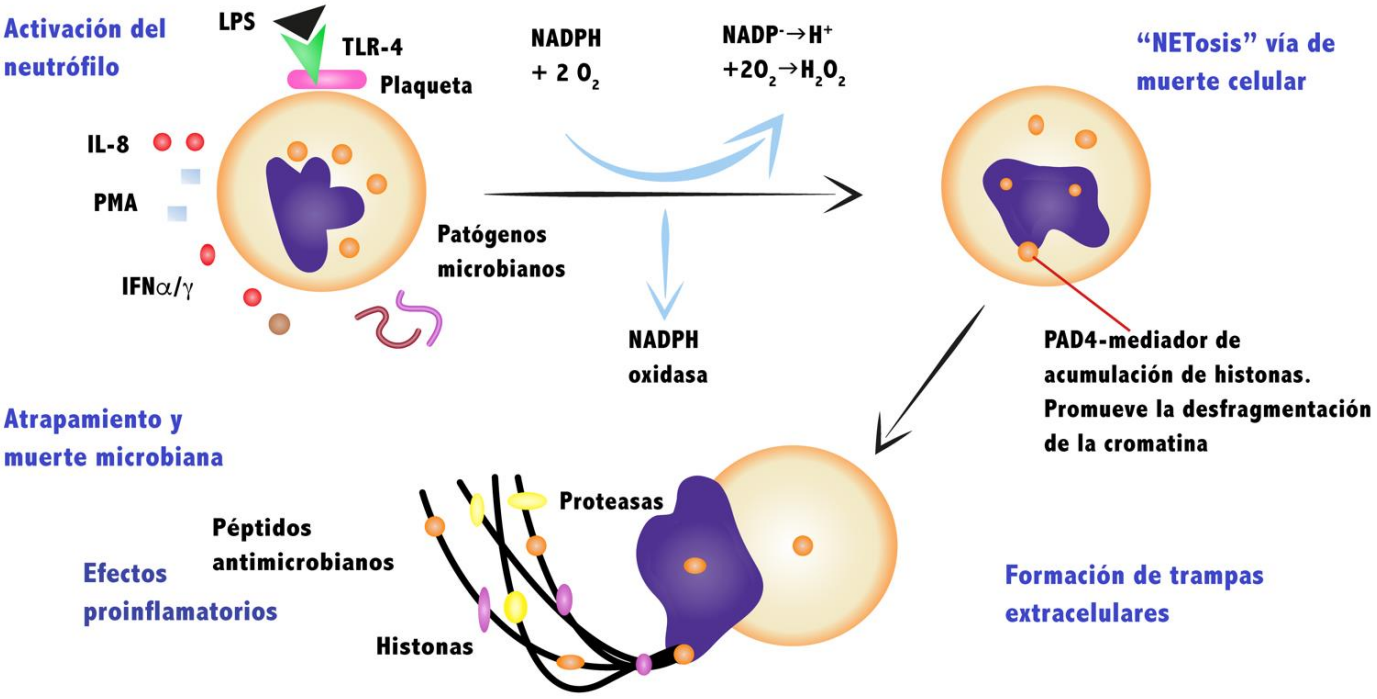


Figura 5. Proceso de NETosis y los efectos de la liberación de NETs. Modificado de Blickwede, 2009.

En estos pacientes se ha observado que el contaje de un subgrupo de neutrófilos, conocidos como neutrófilos de baja densidad (LDNs), estaba aumentado. Los LDNs tienen función proinflamatoria ya que secretan TNF-α e IFN-1, y liberan espontáneamente NETs con DNA mitocondrial oxidada [112]. Estos LDNs pueden aumentar la producción de IFN-1 por células dendríticas plasmacitoides (CDps) y la presentación de antígenos por linfocitos B, favoreciendo la producción de anticuerpos y generando la firma del interferón. Así pues, estos LDNs son reconocidos como células importantes en el inicio y mantenimiento de la autoinmunidad (49). Este tipo de granulocitos han sido asociados a daño endotelial, por lo que podrían jugar un papel importante en la aterosclerosis acelerada de los pacientes con LES [113].

El péptido antimicrobiano LL-37, un componente de las NETs que media la activación de las CDps y que se encuentra en altas concentraciones en los pacientes con LES [114], podría impedir la degradación de las NETs [115]. La producción de NETs y la activación de CDps son los responsables de la producción crónica de IFN-α, que induce la producción de citoquinas proinflamatorias, participando en la alteración del sistema inmune observada en estos pacientes.

Por otro lado, otros estudios han demostrado que las NETs, además de participar en la activación de CDs [115], también participan en la coagulación sanguínea [116] y en la enfermedad

cardiovascular [117]. Como ya se ha comentado, además de constituir un mecanismo de defensa del organismo ante una infección, las NETs favorecen y fortalecen la formación del trombo, ya que actúan como una estructura que atrapa y agrega a las plaquetas y eritrocitos, favoreciendo la formación y deposición de la fibrina. Las cargas negativas de las NETs unen ciertas proteínas plasmáticas como fibrinógeno, fibronectina y factor de von Willebrand (FvW), ayudando a estabilizar el coágulo, activando a proteínas de la fase de contacto, como el Factor XII (FXII), o uniéndose al factor tisular (FT) activando así la fase extrínseca de la cascada de la coagulación [118].

Así pues, las NETs pueden actuar como una espada de doble filo, ya que representan una defensa para el organismo ante agentes infecciosos pero, por otro lado, las proteínas que contienen pueden causar daño tisular potenciado por los efectos de las plaquetas y de las CDps, además de constituir una importante fuente de autoantígenos, lo que desencadena alteraciones en el sistema inmune, perpetuando el ciclo de la disfunción endotelial presente en estos pacientes.

d) Receptores tipo TOLL

Los receptores tipo TOLL (TLR, por sus siglas en inglés), pertenecen a la familia de los RRP, que pueden reconocer las estructuras microbianas conocidas PMAP o moléculas liberadas de células dañadas o muertas, conocidas como *patrones moleculares asociados a daño* (DAMP). La función de estos receptores es distinguir entre lo propio y lo no propio, inducir la producción de citoquinas, activar la respuesta inmune y facilitar la eliminación de restos celulares por fagocitosis [119]. Este tipo de receptores se encuentran en varios tipos celulares relacionados con el sistema inmune, como células presentadoras de antígenos (CPA), CDs, células B y T, mastocitos, NK y neutrófilos, así como en células no relacionadas con éste, como queratinocitos, fibroblastos y células epiteliales [60]

Se han descrito al menos 13 TLRs en mamíferos. Cada TLR reconoce distintos DAMP o PAMPs de la superficie de células inmunes y se encuentran en localizaciones distintas, encontrándose TLR3, TLR7, TLR8 y TLR9 en endosomas y lisosomas y, el resto, en la superficie celular [120].

TLR7 reconoce ARN de cadena simple (scRNA), mientras que TLR9 reconoce autoantígenos asociados a DNA con motivos CpG hipometilados [121, 122] y ambos pueden activar a las CDs y a las células B autorreactivas, rompiendo así la tolerancia inmune [123]. Como en el LES destaca la presencia de autoantígenos nucleares (incluyendo dsDNA, Sm, RNP y Sm-RNP), se cree que la activación de TLR7/9 es crítica en el inicio y en el desarrollo de la enfermedad, mediante la producción de IFN-I y otras citoquinas proinflamatorias. Los ICs presentes en el suero de pacientes

con LES contienen ácidos nucleicos asociados a diferentes proteínas y pueden estimular a las células B y a las CDps a través de TLR7 y TLR9 [124, 125], ya que estas últimas células expresan constitutivamente estos dos receptores y son la principal fuente de IFN-α [126].

Los TLR7 y 9 se encuentran en una posición estratégica, ya que al estar en el interior celular se impide que, en condiciones normales, los receptores sean activados por los ácidos nucleicos circulantes [127, 128]. La estimulación anormal de TLR7 y TLR9 en LES por los propios ácidos nucleicos, de origen apoptótico [129], parecen contribuir a la producción continua de IFN-I, el cuál induce la producción aberrante de autoanticuerpos mediante la estimulación de células B para que se diferencien en células productoras de anticuerpos, se produzca el cambio de clase de inmunoglobulinas y la maduración de CPAs. La sobreexpresión de TLR9 observada en LES sugiere que los ácidos nucleicos liberados durante la apoptosis pueden favorecer la estimulación de linfocitos B mediante este receptor. La expresión tanto de TLR7 como de TLR9 está aumentada en lupus y sus niveles se correlacionan con la expresión de IFN-α [130], por lo que ambos receptores estarían involucrados en la patogénesis del LES [131, 132] y ambos siguen siendo dianas claves para el tratamiento de esta enfermedad [133, 134].

e) La firma del interferón

Los IFNs fueron las primeras citoquinas en ser descubiertas hace más de 40 años. Se encargan de regular la diferenciación, proliferación, apoptosis y supervivencia celular [135]. Se conocen tres tipos (I, II y III) [136] y los tres se han relacionado con el LES, siendo el de mayor importancia el IFN tipo I, ya que promueven la maduración de CDs, la diferenciación de células plasmáticas y la presentación de antígenos por las CPAs [137].

En 1979 se describieron, por primera vez, niveles elevados de IFN-I en esta patología [138], encontrándose elevados en la mayor parte de pacientes adultos y pediátricos con lupus activo [139]. Además, los niveles de IFN-I se han correlacionado con la actividad de la enfermedad [140, 141] y parecen ser claves en el mantenimiento de la inflamación crónica en LES.

Uno de los interferones de tipo I más conocidos es el IFN-α, sintetizado principalmente por CDps vía TLR9 [142, 143], y que tiene diversas funciones en el sistema inmune, como la maduración de CDs, la inducción de la producción de quimiocinas proinflamatorias y la liberación de NETs por neutrófilos, entre otras (Fig. 6) [144]. Preble *y cols.* fueron los primeros en observar niveles aumentados de IFN-α en estos pacientes [145]. El aumento en la síntesis de IFN-α podría explicar, en parte, la alteración inmune observada en esta patología [144], ya que el IFN-α induce la diferenciación de células B autorreactivas a plasmablastos, las cuáles, gracias a la IL-6, se

diferencian después a células plasmáticas secretoras de autoanticuerpos [146, 147]. Es decir, IFN-α disminuiría la tolerancia inmunogénica favoreciendo así la autorreactividad del sistema inmune.

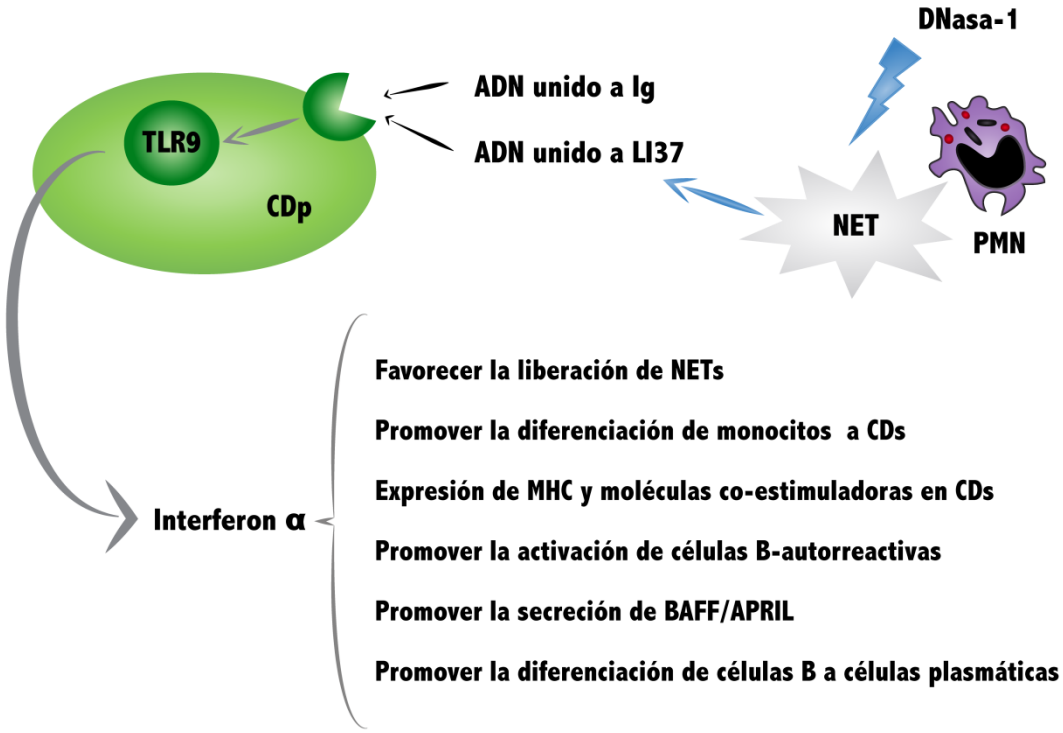


Figura 6. Efecto de las NETs en LES. El aclaramiento ineficiente de las NETs, debido a la presencia de anticuerpos anti-DNasa o a la ineficiencia de la DNasa, favorece la unión de la proteína LL37 al DNA, formando un complejo que puede interaccionar con el TLR-9 intracitoplasmático llevando a la secreción de IFN-α por las CDps. IFN-α tiene un papel fundamental en la patogénesis del LES al inducir la pérdida de tolerancia inmune. Adaptado de Pedram Ahmadpoor *y cols. An Update on Pathogenesis of Systemic Lupus Erythematosus.* Iranian Journal of Kidney Diseases, 2014. 8 (4):171-84.

f) Células dendríticas

Las CDs, descubiertas en los 70 por Ralph Steinman y Cohn Zanvil [148], conectan a la inmunidad innata y a la adaptativa, ya que juegan un papel importante en la regulación de la respuesta inmune. Estas células se distribuyen por todo el organismo para captar antígenos y son consideradas como las principales CPAs, pudiendo activar a las células T naïve.

Los pacientes con LES, con frecuencia, presentan una disminución de CDs en sangre en comparación a los controles sanos que se correlaciona inversamente con la actividad de la enfermedad [149].

Por su origen se conocen, al menos, dos tipos de CDs: las mieloides (CDm) [150] y las CDps, ya mencionadas anteriormente [151]. Se ha visto que las CDm tienen la capacidad de captar material celular apoptótico y necrótico y presentarlas a las células T, produciendo citoquinas proinflamatorias e induciendo la activación de células Th1, Th2 y Th17 [152]. Sin embargo, las CDps no son consideradas como verdaderas CPAs, ya que no presentan moléculas del CMH-II y solo son capaces de ingerir el material apoptótico y necrótico que esté formando complejos inmunes para presentarlo a los linfocitos B, dando lugar a su activación o maduración selectiva [153], y produciendo ellas mismas, grandes cantidades de IFN-I (especialmente IFN-α). Por ello, las CDps son conocidas como la primera fuente de IFN-α y, como ya se ha comentado anteriormente, en pacientes con LES se ha observado una alta concentración de IFN-α, lo que sugiere que este tipo de células también están implicadas en la patogénesis de la enfermedad [154, 155].

2. Sistema inmune adaptativo

La respuesta inmune adaptativa se caracteriza por la especificidad de la respuesta y la memoria inmunológica. Los componentes de la respuesta inmune adaptativa que se han relacionado con LES han sido los linfocitos B y los linfocitos T, así como los anticuerpos.

a) Linfocitos T

Las células T reconocen los antígenos unidos al CMH expresado en las CPAs a través del TCR. En la mayor parte de las células T, el TCR está formado por una cadena α y otra β, unidas mediante un puente disulfuro [156]. En células T tímicas y periféricas, el TCR está formado por una cadena γ y otra δ. El dominio intracelular del TCR es corto, por lo que necesita del clúster de diferenciación 3 (CD3) para la transmisión de señales. El CD3, a su vez, está formado por los heterodímeros δε y γε y el homodímero ζζ del TCR [157].

Se ha visto que en las células T de una gran proporción de los pacientes con lupus, el homodímero ζζ del CD3 está disminuido o es inexistente, debido a diferentes anormalidades moleculares [158-160], y está sustituido por la cadena FcRγ [160, 161], que recluta a la quinasa Syk en lugar de ZAP-70, como ocurre en las células T de controles sanos. La interacción entre FcRγ y Syk es significativamente mayor que la que tendría lugar entre CD3ζζ y ZAP-70 [162, 163], lo que causa un aumento en el flujo de calcio [164], estimulando excesivamente a estas células. Esta anormalidad es específica de la patología y es independiente del tratamiento y de la actividad de la enfermedad [165].

La acumulación de lípidos en la membrana, con dominios ricos de colesterol, soportan al complejo TCR-CD3 y a otras moléculas importantes implicadas en la transmisión de señales [166]. En pacientes con LES, esta agregación lipídica en la membrana de las células T es excesiva, lo que origina una acumulación de TCR en la superficie de estas células [167, 168], produciendo así una activación inapropiada de la cascada de señalización de quinasas y fosfatasas, resultando en una sobrerreactividad de linfocitos T.

b) Linfocitos B

Dos de las características observadas en LES son la tendencia de los linfocitos B a responder de forma exacerbada a la estimulación inmune y la pérdida de tolerancia de estas células [169, 170]. Se ha visto que las células B teóricamente participan en la desregulación inmune observada en el LES a distintos niveles. El por qué y el cómo los linfocitos B se vuelven autorreactivos, pierden la tolerancia, se escapan de los puntos de control centrales y periféricos, se sobreactivan y producen espontáneamente una serie de anticuerpos sigue siendo el punto central en muchas investigaciones clínicas y básicas sobre esta patología.

Uno de los motivos de la estrecha relación entre los linfocitos B y el LES es la producción de una serie de anticuerpos característicos que están claramente implicados en el daño a tejidos. Entre estos anticuerpos se encuentran los anti-DNA que están relacionados con la glomerulonefritis y con la actividad de la enfermedad [171, 172]; anti-β2GP1y anti-cL asociados a casos de trombosis; anti-Ro implicados en el bloqueo cardiaco congénito y desórdenes hematológicos, así como anti-Sm, anti-RNP y anti-La, cuyos mecanismos de acción aún no están claros.

La subpoblación de células B reguladoras (Breg) secretan IL-10 y controlan la proliferación de las células T [173-175]. En relación al lupus se ha publicado que las Breg producen menos IL-10 y tienen comprometida su capacidad supresora [174, 176], aunque su papel en esta patología es muy controvertida ya que existen estudios en los que observan que ratones deficientes en IL-10 desarrollan un lupus más grave [177], mientras que otros grupos de investigación han reportado que el bloqueo de IL-10 con anticuerpos reduce la actividad de la enfermedad [178].

Respecto a la pérdida de tolerancia en células B, estudios GWAS han permitido identificar una serie de genes que predisponen al lupus, entre ellos se incluyen los que codifican para BANK, PTPN2, CD40L, BLK, Lyn, Blimp-1, CD22, AID y FcγIIRB [179, 180]. Estos genes han sido asociados con la alteración de la señalización del receptor de los linfocitos B (BCR), hiperactividad de las células B y la diferenciación de éstas a células plasmáticas.

En relación a las moléculas de superficie, se ha visto que el CD40 en la superficie de linfocitos B activos está disminuido en pacientes con LES, aunque la expresión de CD40L (CD154) está aumentada funcionalmente [181]. La interacción entre CD40 y CD154 está relacionada con la proliferación y secreción de inmunoglobulinas por los linfocitos B periféricos en pacientes con lupus activo [182]. Esta interacción no solo sirve como estimulación a las células T, sino que también promueve la producción de citoquinas tipo Th2, la proliferación de células B, el cambio de isotipo y la activación de la producción de anticuerpos [183-185]. El CD5 se expresa en la membrana de células B y su expresión está disminuida en LES [186].

Fcγ IIRB es un tipo de receptor Fcγ inhibidor de inmunoglobulinas, capaz de reducir las señales del complejo BCR, ya que contiene un dominio de inhibición del inmunorreceptor asociado a tirosina (ITIM) [187] y media la inhibición de las señales de PIP3/PI3K activando a la fosfatasa SHIP y desfosforilando al CD19. En LES se han encontrado células B deficientes en Fcγ IIRB, hecho que aumenta la supervivencia de las células B y contribuye al estado proinflamatorio de la enfermedad [188].

En relación a las citoquinas involucradas en la pérdida de tolerancia de las células B, el estimulador de linfocitos B (BLyS) interviene en el desarrollo y supervivencia de células B mediante su unión a sus distintos receptores: BR3, TACI y el antígeno de maduración de células B (BCMA, por sus siglas en inglés) [189, 190]. APRIL es un homólogo de BLyS, ambos son miembros de la superfamilia de TNF y son críticos para el desarrollo de células B [191]. APRIL y BLyS se expresan en neutrófilos, monocitos, macrófagos, CDs y células B y T y ambos se pueden unir a los receptores TACI y BCMA [192]. En pacientes con LES se han observado niveles séricos elevados de BLys, que protegen a las células B autorreactivas de los procesos de deleción y anergia [193], y se ha observado una correlación con el título de anti-DNA y con la actividad de la enfermedad [194-196]. Al igual que BLys, se han reportado niveles elevados de APRIL en estos pacientes correlacionándose con la actividad de la enfermedad [197, 198]

c) Otras citoquinas

IL-21 es una citoquina producida por las células T foliculares en los centros germinales y es esencial para la selección de las células B en estos mismos centros [199, 200]. Varios grupos de investigación han observado que los pacientes con lupus muestran altos niveles de IL-21 que se correlacionan con la actividad de la enfermedad [201-203].

Aunque las células B aún presentan muchos misterios con respecto a cómo actúan en el LES, es evidente que estas células tienen un papel fundamental en el desarrollo de la enfermedad, ya que

los anticuerpos son las últimas moléculas efectoras en el LES y constituyen una diana terapéutica para su tratamiento.

d) Autoanticuerpos

En el LES se han encontrado anticuerpos contra más de 100 antígenos diferentes, aunque no todos son útiles para el diagnóstico de la enfermedad. En la siguiente tabla se recoge los principales anticuerpos observados en estos pacientes, su frecuencia de aparición, así como su especificidad y las manifestaciones clínicas que producen [204] (Tabla 8).

Autoanticuerpos	Frecuencia	Especificidad	Manifestaciones clínicas
Anti-dsDNA	40-90%	alto	Lupus nefrítico y cutáneo
Anti-Ro	30-40%	Bajo	Lupus nefrítico, cutáneo y afectación cardíaca fetal
Anti-La	12-20%	Bajo	Solo afectación cardiaca fetal
Anti-Sm	5-30%	Alto	Lupus nefrítico
Anti-C1q	40-100%	Alto	Lupus nefrítico
Receptor NMDA	30-50%	Bajo	Neurolupus
Anti-nucleosomas	60-90%	Alto	Lupus nefrítico y cutáneo
Anti-fosfolípidos	20-30%	Alto	Trombosis y abortos

Tabla 8. Anticuerpos más comunes encontrados en pacientes con LES. Adaptado de Sthepen D. *Autoantibodies in systemic lupus erythematosus.* Pediatr Nephrol (2012) 27: 1855-1868.

Los anti-ANA han sido relacionados con vasculitis, manifestaciones cutáneas y fotosensibilidad. Aunque son comunes en los pacientes con LES, no son específicos de esta enfermedad, pues se han encontrado en otras patologías autoinmunes, como en la artritis reumatoide.

Además de ser un marcador de diagnóstico, los anti-dsDNA, también constituyen un criterio de clasificación. Son muy específicos de LES pues están presentes en un alto porcentaje de los pacientes pero en menos de un 0,5% de la población general o pacientes con otras enfermedades autoinmunes [205].

Los anti-nucleosomas se encuentran en otras enfermedades inmunes, como en la esclerosis sistémica, pero su prevalencia en el suero de pacientes con LES es mayor [206, 207]. Se asocian con la gravedad renal [208, 209] y se correlacionan con la actividad de la enfermedad y con los títulos de anti-dsDNA [206, 208].

Aunque no se conoce la contribución exacta de los anti-Sm en LES, son muy específicos de la enfermedad y también constituyen un criterio de clasificación [210], aunque tienen baja sensibilidad porque se encuentran en un bajo porcentaje de pacientes. Han sido relacionados con toxicidad neuronal [211].

Los anti-C1q se han asociado ampliamente con el daño renal en LES [212]. Sus niveles incrementan en brotes de la enfermedad y se normalizan tras la remisión [213].

El N-metil-D-aspartato (NMDA) es un receptor para el glutamato presente en las neuronas. En pacientes con lupus se han encontrado anticuerpos contra el receptor de NMDA, relacionándose con la afectación del sistema nervioso central [214, 215].

Aunque los anti-α-actina no son específicos de la enfermedad se ha demostrado que, cuando están en el suero de pacientes con LES, pueden servir como marcador de daño renal [216-218].

La presencia de anti-Ro o anti-La o de ambos durante el embarazo aumenta un 1-2% el riesgo de que se produzca un bloqueo cardiaco fetal [219]. Los antígenos Ro se exponen en la superficie de los miocitos cardiacos del feto, pero no de la madre, mientras que los anticuerpos anti-Ro de la madre atraviesan la placenta y llegan a su antígeno. Por otro lado, los anti-La se unen a los complejos IgG apoptóticos del corazón del feto. Ambos tipos de anticuerpos se unen a las células apoptóticas del corazón fetal impidiendo su aclaramiento y causando daño tisular [220]. Anti-Ro y anti-La también se consideran marcadores de fotosensibilidad [221, 222].

Los anticuerpos anti-FLs reconocen fosfolípidos con carga negativa, como la anti-cL, el AL y la fosfatidilserina (PS), entre otros y fosfolípidos con carga neutra como el factor activador de plaquetas y el cofactor proteico β2GPI [223], involucrado en la coagulación. A la hora de evaluar la presencia de anticuerpos anti-FLs se suele detectar la presencia de anti-cL, anti-β2GPI y AL. La presencia de anticuerpos anti-FLs se ha relacionado con trombosis venosa y arterial, trombocitopenia y anemia hemolítica, abortos de repetición, úlceras cutáneas y livedo reticularis [224]. Aproximadamente la mitad de los pacientes con LES y anticuerpos anti-FLs desarrollan el síndrome antifosfolípido (SAF), un desorden autoinmune caracterizado por trombosis venosa y arterial de repetición, problemas relacionados con el embarazo, trombocitopenia, anemia hemolítica y niveles elevados de anticuerpos anti-FLs [225].

1.1.6 FÁRMACOS UTILIZADOS EN LES

Actualmente no existe ningún tratamiento etiológico para el LES debido a su origen desconocido, al mecanismo etiopatogénico tan complejo y a las diversas manifestaciones clínicas de esta enfermedad. Por estos motivos resulta difícil establecer un protocolo o una pauta general para el tratamiento de estos pacientes, sino que deben estar adaptados a la situación y gravedad de cada uno de ellos, sin olvidar que los fármacos normalmente empleados en LES no están exentos de efectos secundarios. Por otro lado, diversas instituciones como ACR y EULAR han publicado guías para ayudar en la evaluación y tratamiento de esta enfermedad.

Según la guía de práctica clínica [226], los objetivos del tratamiento son:

- Evitar la aparición de nuevos brotes de la enfermedad
- Prevenir el daño orgánico e irreversible en la enfermedad activa
- Prevenir las comorbilidades y los efectos secundarios del tratamiento
- Mejorar la calidad de vida y aumentar la supervivencia de estos pacientes
- Disminuir la fatiga y el dolor crónico
- Inducir la remisión en el caso de un brote, de forma rápida y duradera en el tiempo

De forma general e independientemente de la actividad y sintomatología clínica de cada paciente, se les recomienda seguir una serie de pautas para evitar la aparición de un brote. Por otro lado, aunque hay pocos fármacos en los que en su ficha técnica figure indicación para el tratamiento del LES, existen una serie de tratamientos convencionales que se han venido usando desde hace décadas. A continuación se exponen las recomendaciones generales y dichos tratamientos.

Recomendaciones higiénico-sanitarias en LES

A estos pacientes, como medidas generales, se les recomienda llevar a cabo una actividad física moderada para tratar la fatiga y la fibromialgia, mantener una dieta equilibrada evitando el sobrepeso y evitar situaciones de estrés. Además, es preferible evitar el alcohol y cesar el hábito tabáquico, ya que puede empeorar las lesiones cutáneas y disminuir la eficacia de los antipalúdicos [227, 228]; protegerse de la luz UV con factor de protección 50, al menos 20 minutos antes de la exposición solar, ya que toda estas situaciones se encuentran asociadas a brotes de LES; por este motivo, la mayor parte de los pacientes suelen necesitar suplementos de vitamina D.

Fármacos convencionales

a) AINEs

Los AINEs se utilizan para el tratamiento sintomático de la enfermedad, como dolor, fiebre, serositis, artritis e inflamación. Su uso debe evitarse en caso de afectación renal.

b) Inmunoglobulinas intravenosas

Las inmunoglobulinas modulan la respuesta inmune al interaccionar con los receptores Fcγ. Entre sus efectos se encuentran la disminución de la producción de autoanticuerpos, la neutralización de anti-DNA, la inhibición del daño producido por el complemento, la modulación de las funciones de los linfocitos T y B, y la producción de citoquinas y antagonistas de citoquinas.

c) Inmunomoduladores

Los inmunomoduladores tienen la capacidad de regular favorablemente el sistema inmune sin aumentar el riesgo de infecciones. En este grupo se encuentran:

- Antipalúdicos (hidroxicloroquina, cloroquina, quinacrina y mepacrina): entre ellos, el más empleado es la hidroxicloroquina, que es considerada como la base del tratamiento de los pacientes con LES, al ser capaz de inmunomodular sin inmunosuprimir, lo que no incrementa el riesgo de infecciones y se recomienda su uso incluso en ausencia de

enfermedad activa [229]. La hidroxicloquina y la cloroquina eran los únicos fármacos aprobados para el tratamiento de LES antes de la aparición del belimumab. Está indicado especialmente para manifestaciones cutáneas y articulares, aunque también se ha descrito su efecto antitrombótico en pacientes con y sin anticuerpos anti-FLs [230, 231]; mejora el perfil lipídico [232], aumenta la supervivencia de estos pacientes [233] y previene el daño orgánico [234, 235] incluyendo los eventos cardíacos, entre otros. Actúa aumentando el pH lisosomal en CPAs, lo que interrumpe el proceso de fagocitosis impidiendo así la presentación de autoantígenos y la secreción de citoquinas proinflamatorias (IL-1, TNF-α…) [236]. Por otro lado, disminuye la señalización por los TLR-3, -7, -8 y -9, reduciendo la activación de CDs y la producción de IFN [237]. La cloroquina, la quinacrina y la mepacrina se emplean en combinación para el tratamiento de las manifestaciones cutáneas refractarias. Aunque la hidroxicloroquina, en una pauta de 200-400 mg/día [238], es el fármaco menos tóxico dentro de este grupo y suele ser bien tolerado y con pocos efectos secundarios se ha descrito, en un número muy bajo de casos, toxicidad ocular a largo plazo como efecto secundario [239].

- Vitamina D: hormona esteroidea implicada en la regulación del calcio y en la hemostasia ósea, además de contar con funciones antiinflamatorias y antiproliferativas. Generalmente, los pacientes con LES tienen una deficiencia o insuficiencia de vitamina D. Esto es debido a que los pacientes suelen presentar diferentes polimorfismos del receptor de la vitamina D3 (VDR), presentes en distintos linajes celulares inmunes. Su deficiencia se relaciona con un aumento en la actividad de la enfermedad, riesgo de trombosis y fatiga. La suplementación con vitamina D disminuye la proteinuria y aumenta los niveles del complemento en estos pacientes [240].

- Deshidroepiandrosterona (DHEA)/prasterona: hormona adrenal precursora de andrógenos y estrógenos producida por las glándulas adrenales. Se secreta como metabolito, DHEA-sulfato (DHEA-s) y tanto DHEA como DHEA-s se convierten posteriormente en esteroides estrogénicos y androgénicos en tejidos periféricos. Mujeres con LES suelen presentar bajos niveles de andrógenos, DHEA y DHEA-s y niveles elevados de estradiol [241]. La suplementación de las pacientes con DHEA se ha relacionado con la regulación de citoquinas inflamatorias, además de reducir la producción de anticuerpos en ratones. En algunos estudios se ha observado que el tratamiento de estas mujeres con 200 mg/día de prasterona oral disminuye la actividad de la enfermedad, es un ahorrador

de esteroides, disminuye los brotes de la enfermedad, y mejora la densidad ósea [242, 243]. El acné y el hirsutismo son los efectos adversos más frecuentes de la prasterona.

d) Corticoides

Los corticoides actúan sobre todos los componentes del sistema inmune, ya que suprimen la producción de citoquinas proinflamatorias e inhiben el reclutamiento de leucocitos gracias a la disminución de la permeabilidad de las células endoteliales y de la producción de moléculas de adhesión. Actúan como antiinflamatorios e inmunosupresores, por lo que se les considera la base en el tratamiento del LES [244]. Actualmente se sigue empleando para los brotes agudos de la enfermedad y estudios han demostrado una reducción en la mortalidad de estos pacientes asociada a su uso, aunque el principal objetivo del tratamiento siempre debe ser la disminución de la dosis de corticoides, ya que están asociados a numerosos efectos secundarios. Se recomienda emplear una dosis de prednisona de ≤7.5mg/día debido a su alta toxicidad y al daño orgánico irreparable que causan [245]. Para evitar esto, la EULAR aconseja dos aproximaciones: a) pulsos de metilprednisolona intravenosa en varias dosis (dependiendo de la gravedad y el peso del paciente) y b) iniciar cuanto antes el empleo de agentes inmunosupresores. Los pacientes con afectación orgánica grave, tras descartar infecciones, se tratan con dosis altas intravenosas de metilprednisolona (250-1000 mg/día durante 3 días) [246].

e) Inmunosupresores

Siguiendo las guías de la EULAR, los inmunosupresores deben reservarse para casos refractarios al tratamiento con antipalúdicos y glucocorticoides, o en situaciones en las que la dosis de esteroides no se puede disminuir por debajo de lo aceptable en tratamientos crónicos. Entre los inmunosupresores más empleados en estos pacientes se encuentran la ciclofosfamida, la azatioprina, el micofenolato y el metotrexato y ninguno de ellos tiene indicación específica para esta enfermedad en España.

- Ciclofosfamida: es un agente alquilante de alta toxicidad que depleciona células B y T e inhibe la producción de anticuerpos, ya que impide la replicación de DNA y la transcripción de RNA [247]. Se utilizaba antiguamente en casos de nefritis lúpica y en manifestaciones del sistema nervioso central (SNC), aunque se ha ido sustituyendo por el micofenolato, por inhibidores de calcineurina, en caso de nefritis, por azatioprina y, en

caso de afectación del sistema nervioso central, por rituximab. Se aconseja una pauta intravenosa de 750 mg/m^2/mes durante 6 meses, pues su uso debe limitarse debido a sus graves efectos adversos como fallo ovárico, cistitis hemorrágica e incremento del riesgo de infecciones, además de ser teratogénico [248, 249].

- Azatioprina: es un análogo de la purina que inhibe la síntesis de ADN y ARN y, por tanto, la proliferación de linfocitos. Suele emplearse en casos de anemia hemolítica y trombocitopenia, así como para el mantenimiento de la respuesta en lupus nefrítico como alternativa al micofenolato en dosis de 1,5-2 mg/kg/día. También se emplea en combinación con corticoides como ahorrador de estos [250] y como alternativa al metotrexato, así como en el embarazo, ya que no afecta al feto [251]. Han demostrado una reducción de brotes de la enfermedad y de mortalidad en pacientes con daño renal y con afectación del SNC [252, 253].

- Metotrexato: antimetabolito inhibidor de la dihidrofolato reductasa que interfiere en la síntesis, reparación y replicación del ADN. Tiene efectos antiinflamatorios ya que favorece la apoptosis de linfocitos activados, reduce el número de linfocitos T proinflamatorios en circulación y de las moléculas de adhesión de células endoteliales y sinoviales, entre otros. El primer ensayo clínico controlado con metotrexato demostró que una dosis de 15-20 mg/semana durante 6 meses era efectiva para controlar la actividad cutánea y articular del lupus, resultando ser también un ahorrador de prednisona [254]. Suele emplearse en pacientes sin afectación orgánica pero con manifestaciones cutáneas y articulares de la enfermedad, favoreciendo la disminución de la dosis de prednisona en estos pacientes. También regulan los niveles de anti-dsDNA y de las proteínas del complemento [255].

  Se recomienda una pauta de 7,5-25 mg/semana por vía oral o intramuscular [256]. Entre sus principales efectos secundarios se encuentran la fibrosis pulmonar y la hepatotoxicidad. Está contraindicado en el embarazo por ser teratogénico.

- Micofenolato: se comenzó a usar en los años 70 para prevenir el rechazo de órganos trasplantados. Entre otros efectos, es citostático, inhibe la proliferación de células T y B, impide la formación de anticuerpos y suprime el reclutamiento de monocitos y linfocitos [257]. Diversos ensayos han mostrado que el micofenolato es efectivo en casos de lupus nefrítico y tiene una eficacia similar a la ciclofosfamida, pero con un perfil de seguridad mejorado. Se aconseja en caso de existir diversas manifestaciones clínicas y que han sido

refractarias a tratamientos convencionales. Está contraindicado en el embarazo por ser teratogénico [258].

El tratamiento de inducción suele realizarse por vía oral a 1 g/día en dos tomas y se va aumentando de forma progresiva en dos semanas hasta una dosis de 2-2,5 g/día, en 2 o 3 tomas diarias [259]. Aunque suele ser bien tolerado, entre los efectos adversos más frecuentes se encuentran náuseas, diarrea y dolor abdominal.

- Inhibidores de la calcineurina: la calcineurina es una enzima dependiente de calcio, responsable de estimular el crecimiento y la diferenciación de células T. Los inhibidores de ésta impiden la traslocación de ciertos factores de transcripción al núcleo, produciendo la inhibición de linfocitos T y la reducción de la producción de citoquinas proinflamatorias [260]. Entre ellos se encuentran:

    - Tacrolimus: es un macrólido inmunosupresor que se une a la proteína intracelular FKBP-12 formando un complejo que inhibe la actividad fosfatasa de la calcineurina impidiendo, en último lugar, la producción de citoquinas proinflamatorias. Tiene mayor potencial molecular que la ciclosporina A y se usó en LES en 1997 por primera vez como tratamiento para el lupus nefrítico, con buenos resultados pero con importantes efectos adversos. La terapia de mantenimiento con tacrolimus durante 5 años mostró más recaídas que aquellos tratados con azatioprina, por lo que la EULAR/ERA-EDTA recomienda que el tacrolimus sea un tratamiento alternativo en casos de lupus nefrítico refractario [261].

    - Rapamicina: al igual que el tacrolimus, la rapamicina se une a la proteína citosólica FKBP12, pero a diferencia de éste, el complejo que se forma inhibe a la diana de rapamicina en células de mamífero (mTOR), inhibiendo la respuesta de IL-2 y, por tanto, bloqueando la activación de las células B y T [262]. La principal ventaja de la rapamicina frente a la ciclosporina y al tacrolimus es que presenta menor incidencia de nefrotoxicidad.

    - Ciclosporina: se une a la proteína intracelular ciclofilina, inhibiendo de esta forma a los linfocitos T-helper con la supresión de la producción de IL-2 y su receptor y

de IFN-γ. El estudio CYCLOFA-LUNE mostró que su efectividad era semejante a la de la ciclofosfamida para preservar la función renal en el lupus nefrítico [263], mientras que el estudio BILAG demostró que la ciclosporina A puede ser un ahorrador de glucocorticoides, pero es menos efectivo que la azatioprina [264].

- Leflunomida: antagonista de la pirimidina aprobada para el tratamiento de la artritis reumatoide. En varios estudios se ha demostrado que la leflunomida es capaz de disminuir la actividad de la enfermedad en pacientes con LES y es eficaz en el tratamiento de lupus nefrítico junto con prednisona [265]. Entre los efectos adversos más frecuentes está la diarrea y reacciones alérgicas cutáneas. Está contraindicado en el embarazo.

- Talidomida: al igual que la lenalidomida, su análogo, tiene una función inmunosupresora, antiangiogénica y antiinflamatoria ya que inhibe la liberación de citoquinas proinflamatorias procedentes de monocitos y modula la acción de otras citoquinas [266]. La talidomina, aunque presenta muy buenos resultados en casos de manifestaciones cutáneas severas en estos pacientes, hay que tener en cuenta que tiene importantes efectos adversos, destacando un aumento del riesgo de trombosis [267, 268].

Terapias biológicas

En las últimas décadas, según se ha ido conociendo más sobre la etiopatogenia del LES, se han desarrollado terapias biológicas para el tratamiento de estos pacientes que actúan directamente sobre dianas específicas de la enfermedad, involucradas en el inicio y perpetuación de la respuesta del sistema inmune que tiene lugar en el LES. Algunas de estas terapias ya se emplean en la práctica clínica habitual, mientras que otras aún se encuentran en ensayo clínico o su empleo en LES se realiza fuera de ficha técnica. Las dianas a las que van dirigidas estas terapias biológicas pueden ser, entre otros: a una línea celular (como células T, B…), a moléculas de activación de distintas líneas celulares, mediadores inflamatorios solubles como inmunoglobulinas, proteínas del complemento, citoquinas, así como dirigidos contra receptores de los distintos mediadores inflamatorios

1. Terapias biológicas con indicación aprobada en LES

a) Diana: células B o células plasmáticas

- Belimumab: anticuerpo monoclonal humanizado que se une específicamente a la forma soluble de BLyS impidiendo así la unión a su receptor en células B. BLyS (estimulador de LB), también conocido como BAFF (factor activador de células B) es una proteína sobreexpresada en pacientes con LES [269], estimuladora de linfocitos B y miembro de la familia del TNF, producida por distintas células del sistema inmune innato. BLyS puede encontrarse en forma soluble o unida a la membrana celular y es esencial para la supervivencia y diferenciación de linfocitos B [194, 270], así como también para la secreción de inmunoglobulinas [190]. El BLyS es capaz de unirse a tres receptores: TACI, el cual se encarga de activar linfocitos B de una forma no dependiente de linfocitos T, está involucrado en la hemostasia de linfocitos B y en el cambio de clase de las inmunoglobulinas; BAFF-R (BR3), que interviene en la generación y supervivencia de linfocitos B maduros, y BCMA, implicado en la diferenciación y supervivencia de células plasmáticas. Se ha visto que los niveles de BLyS se correlacionan con la actividad de la enfermedad [194, 197] y con los niveles de autoanticuerpos en los pacientes con LES [271].

  El belimumab fue el primer fármaco aprobado por la Agencia Europea del Medicamento en el 2011, para el tratamiento de LES tras los resultados de los ensayos de fase III BLISS 52 y BLISS 76 [272, 273], donde demostró su seguridad y efectividad, y en donde se probó el fármaco a dosis de 10 mg/kg como coadyuvante en pacientes adultos con anti-ANA y/o anti-dsDNA positivos y enfermedad activa (SELENA-SLEDAI ≥ 6). En estos ensayos se excluyeron a pacientes con manifestaciones renales o del SNC, por lo que no se conoce la efectividad del belimumab en estos grupos. Actualmente se está llevando a cabo un ensayo en fase III (BLISS-LN, NCT01639339) para estudiar la utilidad de este fármaco en pacientes con lupus nefrítico, cuyos resultados hasta el momento están siendo positivos.

  Se recomienda comenzar el tratamiento con belimumab a una pauta de 10, 14 y 28 días y, posteriormente, a intervalos de 4 semanas. Si después de 6 meses desde el día el comienzo del tratamiento no se ha observado mejoría, se debería valorar la interrupción del tratamiento [274].

2. Terapias biológicas sin indicación aprobada en LES

a) Diana: células B o células plasmáticas

- Rituximab: anticuerpo quimérico monoclonal contra la proteína CD20, que se expresa en todas las etapas de las células B, pero desaparece una vez que maduran a células plasmáticas; por tanto, promueven la depleción por citotoxicidad mediada por el complemento y los anticuerpos, mediante la porción Fc, induciendo la apoptosis de células $CD20^{+}$.

  El rituximab no tiene indicación específica para LES, pero ensayos clínicos han mostrado buenos resultados en pacientes con enfermedad recalcitrante, incluso con manifestaciones renales, hematológicas y cutáneas. Aunque los resultados del ensayo EXPLORER (lupus sin afectación renal o extranefrítico) y LUNAR (lupus nefrítico) no alcanzaron unos resultados satisfactorios [275, 276], estudios retrospectivos han probado su eficacia para disminuir la dosis de corticoides cuando se combina ciclofosfamida y rituximab [277], así como también en lupus nefrítico [278]. Actualmente se utiliza fuera de ficha técnica en casos graves de anemia hemolítica y trombocitopenia, como alternativa a la azatioprina. Los efectos adversos del rituximab son muy poco frecuentes pero graves, como infecciones oportunistas y leucoencefalopatía. Debido a su naturaleza quimérica, el 10% de los pacientes tratados con rituximab han experimentado reacciones alérgicas o anafilácticas.

  Aunque la pauta de tratamiento en pacientes con LES no está definida, según los ensayos clínicos realizados se aconseja administrar una dosis de 1 g los días 1 y 15 y repetir los ciclos cada 24 semanas [275].

b) Diana: Moléculas co-estimuladoras de células B y T

- Abatacept: proteína de fusión formada por la región Fc de la inmunoglobulina IgG1 (CTLA-4-Ig) y el dominio extracelular de CTLA-4 (CD152). El CD28 es un ligando coestimulador de células B que interacciona con los receptores CD80 y CD86. El abatacept actúa uniéndose al CD80 y al CD86 con mayor afinidad que CD28, bloqueando la interacción co-estimuladora entre linfocitos B y T [279], disminuyendo la producción de células B productoras de anticuerpos [280], controlando la proliferación de linfocitos T y

disminuyendo la producción de citoquinas proinflamatorias (TNF-α, IL-2, IL-4, IL-5, IL-6, IL-17 e IFN-γ) producidas por linfocitos T activados [281]. Además, el abatacept también inhibe la formación de células T-helper foliculares e induce el cambio de células T naïve $CD4^+CD25^-$ a células T reguladoras $CD4^+CD25^+$ [282], e inhibe la migración de los monocitos lo que tiene efectos antiinflamatorios directos [283]. Un estudio realizado por Daikh *y cols.* en modelos murinos de lupus nefrítico mostraron cómo el tratamiento de estos con CTLA-4-Ig inhibía la producción de autoanticuerpos [284] y, al combinar el tratamiento con ciclofosfamida, se observó una reducción de las manifestaciones renales de la enfermedad y la prolongación de la supervivencia [285]. El abatacept fue aprobado por primera vez para el tratamiento de la artritis reumatoide refractaria a tratamientos convencionales y aunque actualmente no está aprobado para LES, varios ensayos clínicos han demostrado su seguridad en estos pacientes, obteniéndose resultados prometedores en pacientes con y sin compromiso renal [286, 287].

c) Diana: citoquinas inflamatorias y sus receptores

- Infliximab (Remicade): anticuerpo monoclonal IgG1 quimérico humanizado dirigido contra la proteína TNF-α, tanto en su forma soluble como a la forma transmembrana [288], por lo que inhibe la función de ésta durante su participación en el proceso inflamatorio e inmunitario del organismo. Los ensayos clínicos llevados a cabo con infliximab han sido escasos y con un número bajo de pacientes incluidos, observándose que la administración de infliximab junto con inmunosupresores mejoraba la nefritis lúpica [289] y que los pacientes mostraban una mejoría según el SLEDAI en comparación al tratamiento estándar, pero no hubo cambios en la dosis de corticoides, en el SLICC/ACR, ni en los niveles de proteinuria, de anticuerpos y de proteínas del complemento [290]. En otros estudios se ha observado una disminución en la actividad clínica del LES pero con aparición o aumento de anti-DNA y anti-cL [291, 292].

- Tocilizumab: anti-IL-6R humanizado que actúa bloqueando la unión de la IL-6 a su receptor, tanto en su forma soluble (sIL-6R) como anclado a la membrana (mIL-6R), lo que provoca una reducción de la síntesis de anticuerpos y anti-dsDNA [293, 294]. La IL-6 es producida por macrófagos, neutrófilos, CDs y por células T $CD4^+$ y se asocia a señales de inflamación, como la fatiga o la fiebre [295]. Fue aprobado para el tratamiento de la enfermedad de Castleman y de la artritis reumatoide, por lo que una indicación

terapéutica potencial es el LES, ya que varios trabajos han demostrado una correlación entre los niveles de IL-6, la actividad de la enfermedad y el incremento de los títulos de anti-DNA [296, 297]. En un ensayo de fase I con dosis escaladas de tocilizumab se observó una mejoría clínica y serológica de la inflamación en estos pacientes, reflejada en una disminución en marcadores de inflamación y en los niveles de anti-dsDNA y en la mejora de los niveles de hemoglobina y de albúmina sérica, lo que demostró una neutralización efectiva de IL-6 en estos pacientes [298]. El uso de tocilizumab puede ser una alternativa a considerar en el caso de los pacientes refractarios a corticoides, citostáticos y rituximab, aunque su eficacia debe ser aún estudiada mediante más ensayos clínicos abiertos y controlados [299].

- Etanercept: es una proteína de fusión dimérica recombinante formada por la fusión de dos copias del receptor p75 de TNF unida a la porción Fc de la IgG1 humana, capaz de unirse tanto a TNF-α como a la linfotoxina circulante, impidiendo que actúen sobre los receptores celulares y bloqueando la respuesta inflamatoria celular, modulando los efectos de otras moléculas reguladas o inducidas por TNF [300]. Su uso fue aprobado por primera vez para la artritis reumatoide aunque se usa fuera de ficha técnica para LES, enfermedad de Behçet y esclerosis sistémica, entre otros. Aunque en la literatura se han descrito varios casos de pacientes con LES tratados con etanercept [301-303], aún se tienen pocos datos sobre sus riesgos en estos pacientes.

3. Moléculas en ensayo clínico

Aunque la patogénesis del lupus aún no se conoce completamente, actualmente existen más de 70 terapias en ensayo clínico dirigido a distintas dianas de la enfermedad. Entre estas dianas se encuentran las células B o plasmáticas, las moléculas coestimuladoras de células B/T, citoquinas inflamatorias y sus receptores, IFNs, señales intracelulares y CDps (Fig. 7). A continuación se expone una tabla que recoge algunos de los fármacos en ensayo y su diana terapéutica (Tabla 9) [304, 305].

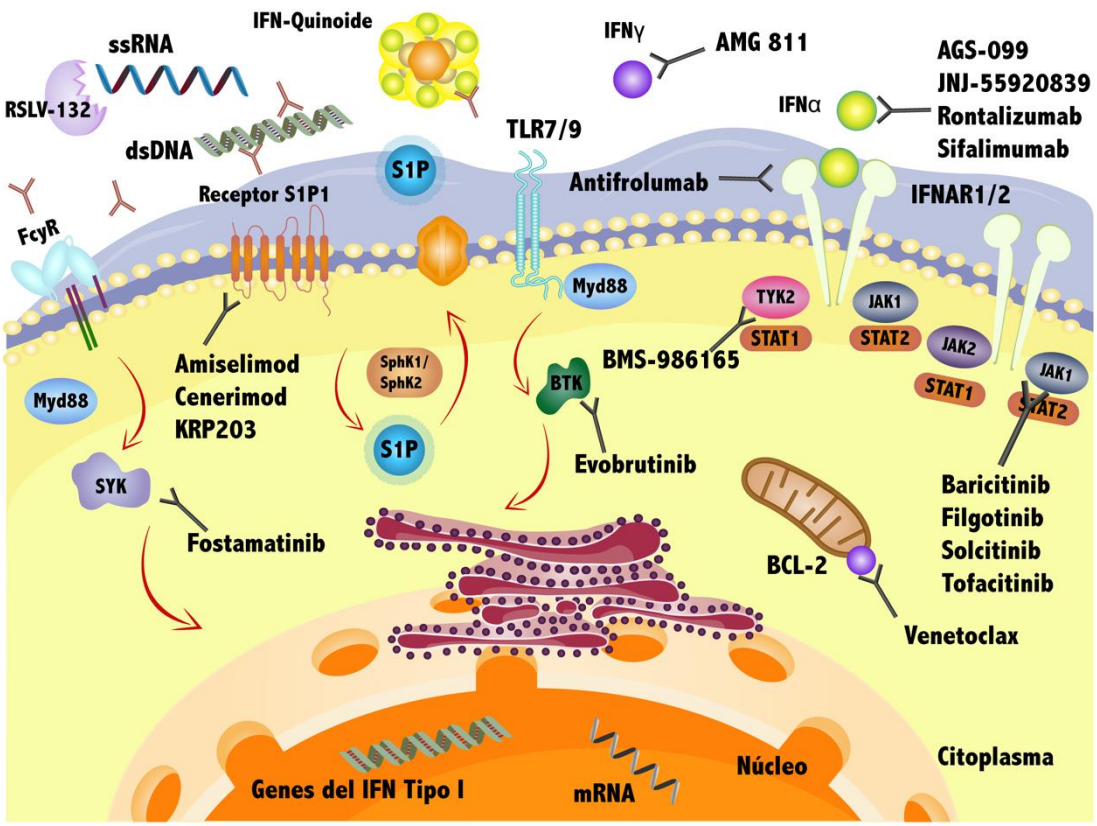


Figura 7. Diana de las distintas moléculas en ensayo clínico. Adaptada de Felten Renaud et al. *Advances in the treatment of systemic lupus erythematosus: from back to the future, to the future and beyond*. Joint bon espine (2018)

Fármaco	Diana
Células B o plasmáticas	
Obinutuzumab	Anti-CD20
TRU-015	Anti-CD20
Ocrelizumab	Anti-CD20
SM03	Anti-CD22
Milatuzumab	Anti-CD74
Tabalumab	Anti-BAFF
Atacicept	Anti-BAFF
AMG 570	Anti-BAFF
RC-18	Anti-TACI
Ixazomib	Proteasoma

Fármaco	Diana
Moléculas coestimuladoras de células B/T	
Abatacept	Anti-CTLA
RG2077	Anti-CTLA
BI655064	Anti-CD40
Lulizumab	Anti-CD28
Teralizumab	Anti-CD28
AMG 577	Anti-ICOSL
MEDI570	Anti-ICOSL
Dapirolizumab	Anti-CD40L
Citoquinas proinflamatorias	
Etanercept	Anti-TNFα
Sirukumab	Anti-IL-6
PF-04236921	Anti-IL-6
MRA 003 US	Anti-IL-6R
Ustekinumab	Inhibidor de IL-12/23
Vobarilizumab	Anti-IL-6R
Brentuximab	Anti-CD30
Emapticap	Anti-CCL2
PF-06835375	inhibidor de citoquinas
Interferones y sus receptores	
Rontalizumab	Anti-IFNα
Sifalimumab	Anti-IFNα
AGS-009	Anti-IFNα
JNJ-55920839	Anti-IFNα
Anifrolumab	Anti-IFN-1(R)
Señales intracelulares	
Tofacitinib	Inhibidor de JAKs
Filgotinib	Inhibidor de JAKs
Baricitinib	Inhibidor de JAKs
Solcitinib	Inhibidor de JAKs

Fármaco	Diana
BMS-986165	Inhibidor de TYK2
Evobrutinib	Inhibidor de BTKi
KRP203	Inhibidor de esfingosina-1-fosfato
Cenerimob	Inhibidor de esfingosina-1-fosfato
Amiselimod	Inhibidor de esfingosina-1-fosfato
Células dendríticas plasmacitoides	
Talacotuzumab	Anti-CD123
Venetoclax	Inhibidor de BCL-2
BIIB059	Anti-BDCA-2(R)

Tabla 9. Distintas moléculas en ensayo clínico para el tratamiento del LES y sus dianas.

1.1.7 MANIFESTACIONES CLÍNICAS Y SU TRATAMIENTO

Al tratarse de una enfermedad inflamatoria y multisistémica, en la que diversos órganos pueden verse afectados de forma aislada o de manera simultánea, no existe un cuadro clínico característico en esta patología [306], pudiendo observar manifestaciones clínicas muy dispares tales como afectación musculoesquelética, mucocutánea, cardiaca, renal, pulmonar y gastrointestinal, entre otros (Fig. 8).

Manifestaciones generales

Las manifestaciones generales del LES son muy inespecíficas, pudiendo ser confundidas con síntomas de otras enfermedades autoinmunes o infecciones, anormalidades endocrinas, fatiga crónica y fibromialgia [307].

La fiebre suele ser característica en un brote de la enfermedad, que puede estar presente tanto en el inicio como en el transcurso de ésta y debiendo descartar la presencia de infecciones concomitantes, tumores o el efecto de algunos fármacos en el organismo.

La fatiga es una manifestación que cursa de forma independiente a la sintomatología clínica y serológica del LES y que puede llegar a ser incapacitante; llegando a relacionarse con trastornos

depresivos en este tipo de pacientes. La anorexia, la astenia, la pérdida de peso y los dolores de cabeza pueden preceder a síntomas clínicos más específicos de la enfermedad.

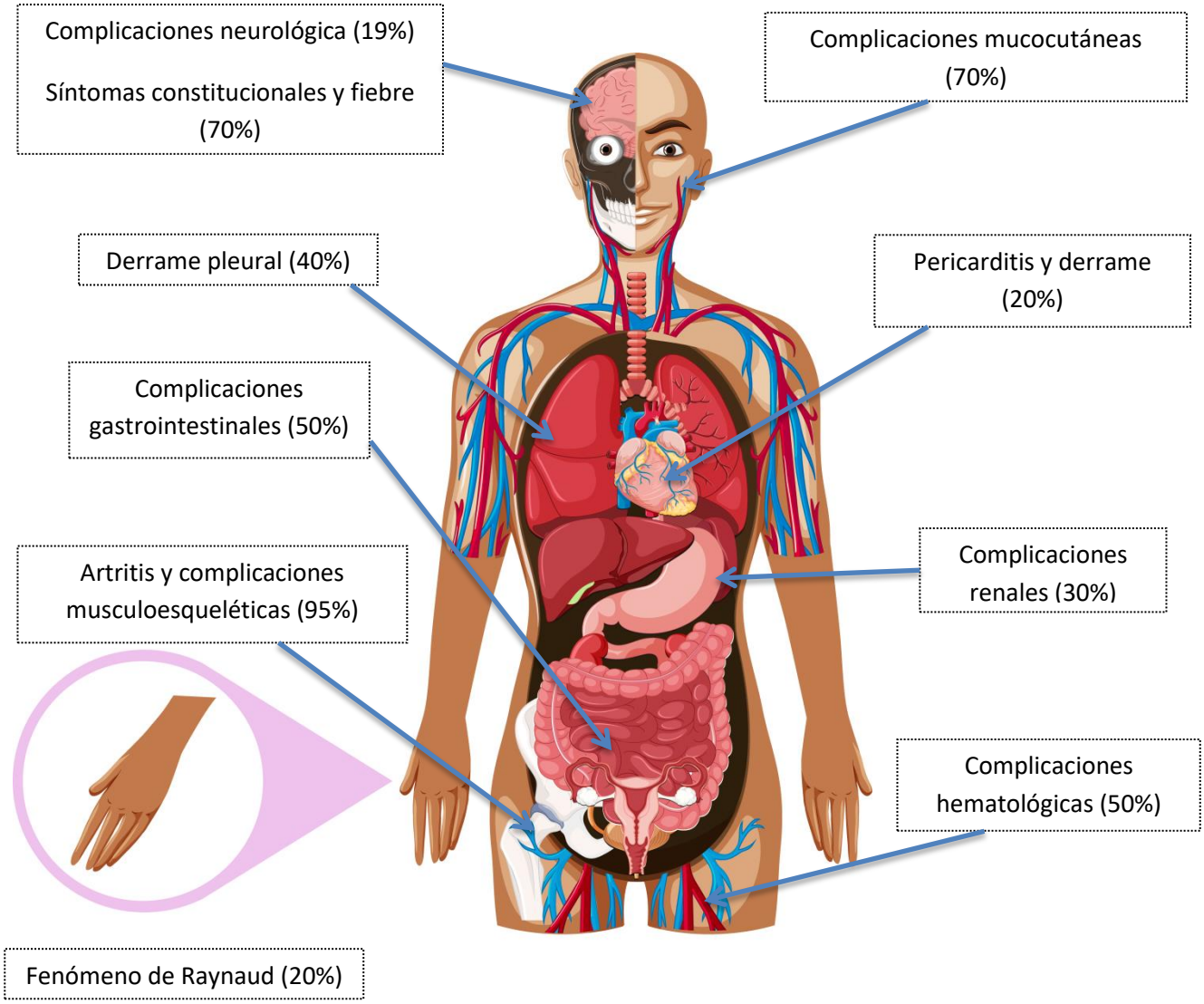


Figura 8. Manifestaciones clínicas encontradas en pacientes con LES y su frecuencia de aparición.

Las manifestaciones generales más frecuentes son leves y moderadas, que suelen remitir con antipalúdicos o corticoides a dosis bajas en unos días. En caso de manifestaciones graves, cuando el paciente no responde a los tratamientos previos, es común el uso de la azatioprina o el

metotrexato y, si no hay respuesta en 2-3 meses, el belimumab suele dar buenos resultados. En casos refractarios, es necesario recurrir a otras terapias biológicas.

Manifestaciones musculoesqueléticas

Las manifestaciones articulares aparecen en el 95% de estos pacientes y son uno de los primeros síntomas clínicos del LES. Pueden ir desde artropatías hasta poliartritis, aunque no suele ser deformante ni erosiva.

En un 10% de los pacientes con enfermedad prolongada puede aparecer la artropatía de Jaccoud y deformidad en cuello de cisne, aunque ambas son infrecuentes.

La afectación muscular se ha descrito en un 30-50% de los pacientes en forma de mialgia, dolor y debilidad muscular, pudiendo ser una manifestación propia del LES o secundaria a la artritis, a una miositis o al empleo de ciertos medicamentos, como los corticoides.

Estas manifestaciones suelen tratarse con AINES, prednisona e hidroxicloroquina como terapias de primera línea. Cuando no mejoran, es común añadir una segunda línea de tratamiento inmunosupresora como metotrexato [254, 308-310] y micofenolato, incluso azatioprina, leflunomida [265, 311] y ciclosporina.

El belimumab a dosis de 1-10 mg/kg también es efectivo para mejorar estos síntomas. Rituximab, en combinación con esteroides, ha demostrado dar buenos resultados en manifestaciones musculoesqueléticas severas. Se aconseja usar abatacept solo en pacientes refractarios a tratamientos convencionales [312].

Manifestaciones mucocutáneas

Las manifestaciones mucocutáneas en estos pacientes son muy variadas, pudiendo clasificarse en específicas o no específicas de la enfermedad. Las lesiones específicas se pueden dividir en agudas, subcutáneas y crónicas, destacando el característico *rash malar* o alas de mariposa, referido al eritema doloroso y elevado de distribución malar, que no deja cicatriz y propiciado, principalmente, por la luz solar.

Entre las lesiones no específicas pero que son frecuentes se encuentran la fotosensibilidad, la alopecia, las aftas orales y los nódulos subcutáneos, pudiendo observase también livedo reticularis, tromboflebitis e infarto periungueal.

Manifestaciones hematológicas

Varios estudios han demostrado la presencia de anormalidades hematológicas en LES. La anemia es la más frecuente, observándose en más del 50% de los pacientes, siendo la más frecuente la ferropénica, pudiendo deberse al tratamiento con AINES, aunque también se ha descrito la anemia hemolítica inmune.

La presencia de anticuerpos antiplaquetarios en estos pacientes puede causar trombocitopenia moderada y púrpura trombocitopenia inmune, aumentando la morbilidad y mortalidad en LES.

La leucopenia, generalmente con linfopenia, puede deberse a la destrucción de células blancas por anticuerpos, a una disminución de la producción en médula ósea y a la activación del complemento, aunque también fármacos empleados en LES pueden causar un descenso en el número de estas células, aumentando la predisposición a infecciones. Es característica de la enfermedad y uno de los criterios de clasificación de LES.

Diversos grupos han demostrado que los pacientes con LES tienen mayor riesgo de sufrir neoplasias hematológicas, especialmente linfoma de non-Hodgkin, seguido de leucemia y mieloma múltiple.

Por otro lado, los niveles de colesterol y de triglicéridos suelen estar aumentados en LES. La presencia de anticuerpos anti-ANA, anti-DNA, anti-histona, anti-Smith, anti-La/Ro y anti-FLs son altamente frecuentes en estos pacientes, así como anticuerpos contra leucocitos y eritrocitos.

Los anticuerpos anti-FLs están relacionados con eventos trombóticos arteriales y venosos, trombocitopenias y complicaciones obstétricas en mujeres embarazadas. Un alto porcentaje de las complicaciones de esta enfermedad se deben directamente a la trombosis.

Manifestaciones neuropsiquiátricas

Las manifestaciones neuropsiquiátricas en LES son complejas y variadas. Estudios prospectivos sitúan la incidencia de estas manifestaciones en el 19% de los pacientes [313], pudiendo afectar a cualquier parte del cerebro, a los nervios craneales y periféricos, las meninges o la médula espinal.

En 1999, el ACR creó una nomenclatura para recoger los síntomas neuropsiquiátricos del lupus y se registró los 19 síndromes que contaban con criterios de diagnóstico, dependiendo de si afectaban al SNC o al periférico [314] (Tabla 10).

Sistema Nervioso Central	Sistema Nervioso Periférico
Meningitis aséptica	Poliradiculopatía desmielinizante
Enfermedad cerebrovascular	Aguda (Guillain Barré)
Síndrome desmielinizante	Desórdenes autonómicos
Cefalea (incluyendo migraña)	Mononeuropatía
Trastorno del movimiento	Miastenia Gravis
Mielopatía	Neuropatía Craneal
Trastorno convulsivo	Plexopatía
Estado confusional agudo (delirio)	
Desórdenes de ansiedad	
Disfunción cognitiva	
Psicosis	
Trastorno del ánimo	

Tabla 10. 19 Síndromes neuropsiquiátricos definidos por el ACR observado en pacientes con LES. Adaptado de Arthritis & Rheumatology 1999; 42:599-608

Además, también se han observado anormalidades neuroanatómicas en pacientes que presentaban este tipo de afectación, observándose distrofia cortical, infartos, desmielinización y, con baja frecuencia, vasculitis pura.

Este tipo de manifestaciones se ha relacionado con la presencia de anti-FLs, debido al riesgo de eventos vasculares y alteraciones cognitivas [313], especialmente los anti-cL con migrañas, psicosis y depresión, entre otros [315].

El tratamiento de las diferentes manifestaciones neuropsiquiátricas varía dependiendo de si éstas son inflamatorias, trombóticas o de ambos tipos.

La EULAR recomienda tratar las manifestaciones inflamatorias con inmunosupresores (principalmente con azatioprina y ciclofosfamida) y corticoides. En caso de manifestaciones severas como psicosis, mielitis o estado confusional se aconseja una terapia de inducción con altas dosis de corticoides y ciclofosfamida intravenosa, seguido de un tratamiento inmunosupresor para el mantenimiento de la remisión [316]. En caso de depresión, disfunción cognitiva y manifestaciones cerebrovasculares se ha de seguir el mismo tratamiento que el indicado para la población general. Si las manifestaciones son trombóticas es necesario el uso de anticoagulantes o antiagregantes, especialmente en aquellos pacientes con anticuerpos anti-FLs [317].

Por otro lado, el rituximab ha dado buenos resultados para el tratamiento de déficits cognitivos y psicosis [318]; las inmunoglobulinas intravenosa, inmunoadsorción y la plasmaféresis deberían reservarse para casos refractarios no causados por SAF o la aterosclerosis [319].

Manifestaciones oculares

Las diferentes estructuras oculares pueden ser dianas de la enfermedad [320, 321], apareciendo este tipo de manifestaciones en un tercio de los pacientes [322]. La queratoconjuntivitis es la manifestación ocular más común y ocurre en el 25% de los pacientes.

Las escalas SLEDAI y SLEDAI-2K incluyen este tipo de manifestaciones como criterio de enfermedad activa [323]. Un estudio demostró cómo el SLEDAI correlacionaba con la presencia de lesiones oculares [324].

Por otro lado, ciertos fármacos antimaláricos empleados en el tratamiento del LES causan retinopatía y toxicidad ocular, como la cloroquina y la hidroxicloroquina [325]. Por ello es esencial que los pacientes que tengan alguna manifestación ocular y/o estén en tratamiento con estos

antipalúdicos sean controlados de forma regular y tratados correctamente para evitar complicaciones posteriores [326].

Manifestaciones obstétricas

Algunos estudios han demostrado que la fertilidad de mujeres con LES está disminuida, bien por los brotes de la enfermedad, por el propio tratamiento inmunosupresor o por el daño renal. Estas pacientes además, presentan un alto riesgo de sufrir abortos espontáneos, prematuridad y restricción del crecimiento fetal.

Se ha registrado un 2-3% de mortalidad materna en aquellos pacientes con LES activo, especialmente relacionado con nefropatía lúpica [327].

En pacientes con anticuerpos anti-FLs, especialmente de tipo anti-cL y AL, el riesgo de aborto espontáneo se ve incrementado y su presencia se ha relacionado con el síndrome HELLP y la preeclampsia [327, 328].

La afectación pulmonar se puede manifestar de forma leve o aguda, pudiendo tener muchas complicaciones. Pueden afectar a la vía aérea superior, al parénquima pulmonar y a su vasculatura, a la pleura e, incluso, a la musculatura respiratoria.

Entre las manifestaciones pulmonares encontramos la neumonía infecciosa, hipoxemia aguda reversible, la neumonitis lúpica aguda, pleuritis, hemorragia alveolar, hipertensión, tromboembolismo pulmonar y debilidad de la musculatura respiratoria [329-332].

Existe una prevalencia de afectación pulmonar a lo largo de la enfermeda entre el 5-90% [333], dependiendo del diseño del estudio en el que se haya hecho esta evaluación y de los criterios sintomatológicos o histopatológicos empleados para el diagnóstico del LES [329, 334].

La enfermedad cardiovascular es la segunda causa de muerte, tras infecciones, en pacientes con lupus [335]. La cardiopatía isquémica en estos pacientes tiene una prevalencia de 3,8-16% [336, 337], 10 veces más que en la población general [338], y en mujeres con LES en edad reproductiva el riesgo es 50 veces mayor [339]. Un 15 % de los pacientes con LES sufren infarto de miocardio [340, 341] y cerca del 20-30% de las muertes ocurridas en los pacientes con LES se deben a la enfermedad cardiovascular (ECV) [342-344].

Este aumento en el riesgo de infarto de miocardio comparado con la población general no se puede explicar completamente por los factores de riesgo tradicionales (tabaco, obesidad,

hipertensión, dislipemia y diabetes), lo que implica la existencia de otros factores inherentes a la enfermedad que contribuyen a los problemas cardiovasculares en estos pacientes (Fig. 9).

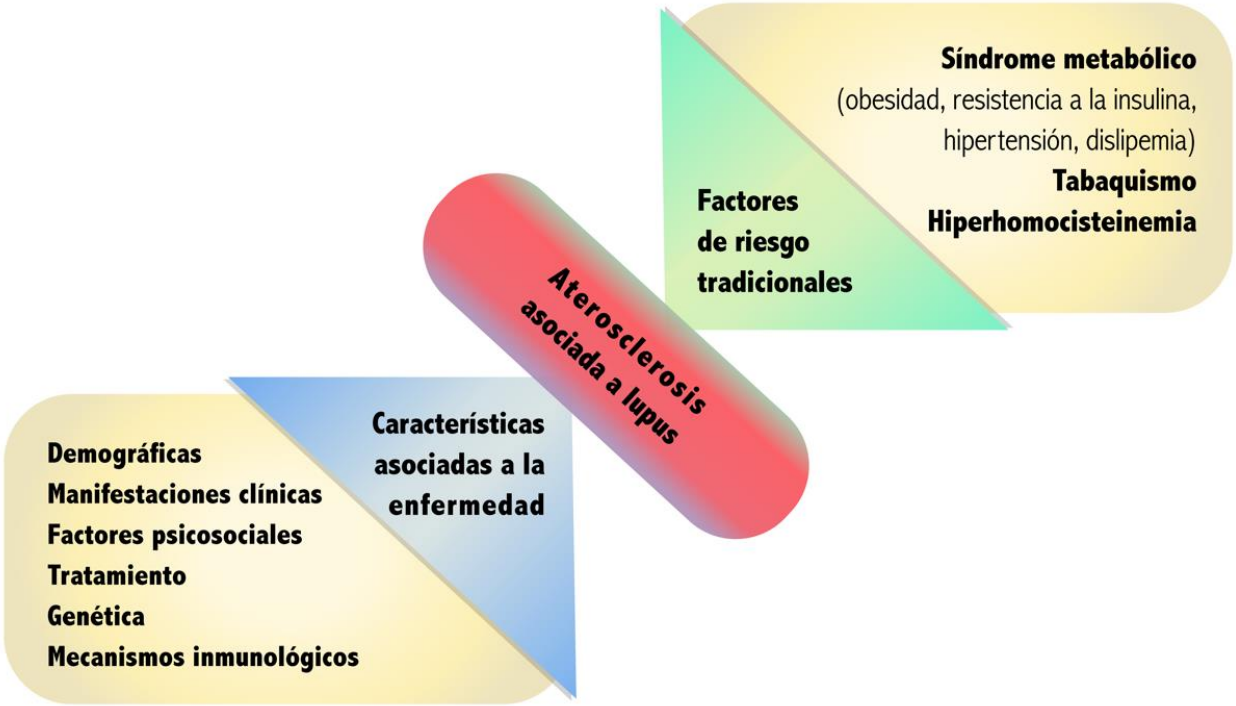


Figura 9. Factores de riesgo aterosclerótico en LES. La figura recoge las características asociadas a la enfermedad y los factores de riesgo tradicionales relacionados con la aterosclerosis observada en pacientes con LES.

En pacientes donde hay existencia de anti-cL se han observado anomalías valvulares llamadas endocarditis de Libman-Sacks.

Para evitar las manifestaciones de este tipo es importante que los pacientes lleven un estilo de vida sano, realizando actividad física y manteniendo hábitos saludables.

Manifestaciones gastrointestinales

Las úlceras orales son comunes en LES y constituyen un criterio de diagnóstico de la enfermedad. El dolor abdominal observado en pacientes con LES puede estar relacionado con la enfermedad activa, incluso puede llegar a ser peritonitis, isquemia abdominal por vasculitis, pancreatitis o trombosis. La propia actividad de la enfermedad eleva las transaminasas, las cuales suelen volver a la normalidad después del uso de corticoides [345].

Manifestaciones renales

Las manifestaciones renales de la enfermedad pueden ir desde un compromiso renal hasta un síndrome nefrótico con deterioro del filtrado glomerular potencialmente fatal.

Para el tratamiento de la nefritis lúpica, la biopsia renal es esencial, ya que hay que tener en cuenta el subtipo histopatológico de nefritis, grado de actividad y la cronicidad de la lesión renal. El objetivo del tratamiento de la nefropatía lúpica es inducir la remisión parcial o completa (Tabla 11) y, posteriormente, el mantenimiento de ésta.

Remisión parcial	Remisión completa
Paciente con una proteinuria basal ≥3,5 g/24h, que descienda a <3,5g/24h. Pacientes con una proteinuria basal <3,5g/24h, que se produzca una reducción de <50% en comparación con la basal. En ambos casos, que se produzca una mejoría de los valores basales del filtrado glomerular.	Filtrado glomerular ≥60 ml/min/1,73 m^2 (así como descenso a valores iniciales o ±15% del valor inicial en pacientes con un filtrado glomerular de <60 ml/min/1,73 m^2). Valores de proteinuria ≤ 0,5g/24h. Un sedimento inactivo (≤5 leucocitos, ≤5 hematíes y 0 cilindros hemáticos). Concentración de albúmina sérica > 3 g/d.

Tabla 11. Criterios de remisión parcial y completa. Adaptada de la Guía de Enfermedades Autoinmunes Sistémicas de la Sociedad Española de Medicina Interna, 2011.

Vasculitis y manifestaciones vasculares. Síndrome antifosfolípido

Para el tratamiento de los síntomas vasculares leves, como el fenómeno de Raynaud, se aconseja evitar fármacos vasoconstrictores (cocaína, betabloqueantes, anfetaminas), el tabaco y el alcohol; no suelen necesitar tratamiento farmacológico, pero pueden emplearse antipalúdicos. El tratamiento antiagregante es obligatorio en aquellas mujeres con SAF secundario para prevenir los abortos.

1.2 LAS PLAQUETAS

1.2.1 GENERALIDADES DE LAS PLAQUETAS

Las plaquetas o trombocitos son pequeñas células discoides anucleadas derivadas del citoplasma de los megacariocitos (MKs) y cuya producción es estimulada por la trombopoyetina (TPO). El cuerpo humano produce y elimina 10^{11} plaquetas diariamente con el fin de mantener un contaje normal y estable de éstas entre 150000 y 400000 plaquetas/mm^3 [346], pues el balance entre la producción y la destrucción de las plaquetas es esencial no solo para la hemostasia, sino para mantener un balance saludable en la respuesta inmune.

Así pues, las plaquetas representan la segunda entidad más numerosa en la circulación, por detrás de los eritrocitos [347]. Tienen un tamaño de 0,5 a 2,5 µm, el volumen plaquetario medio fluctúa entre 7 y 9 fL y tienen una vida media de 7-10 días, tras el cual son destruidas por los macrófagos de la médula ósea, del sistema reticulohistiocitario del bazo y del hígado.

1.2.2 MEGACARIOPOYESIS Y TROMBOPOYESIS

La megacariopoyesis se define como el proceso de diferenciación de la línea megacariocítica, desde las células hematopoyéticas iniciales más indiferenciadas hasta el producto final, las plaquetas; mientras que el término trombopoyesis define el proceso de liberación de plaquetas a partir del MK maduro.

Las plaquetas provienen del citoplasma de los MKs, grandes células (50-100 µm) [348] que se encuentran en la médula ósea, y que se someten a un proceso conocido como endomitosis o endorreduplicación [349], una replicación del material nuclear sin división celular, creando así una célula con un núcleo multilobulado. El proceso de diferenciación del MK continúa y en él intervienen varias citoquinas, principalmente la trombopoyetina (TPO) pero también IL-6, IL-3, IL-4, IL-6 e IL-11 y el factor de célula madre. El MK da lugar a las proplaquetas que, finalmente, se convierten en plaquetas y se liberan directamente a la circulación

La TPO es la hormona responsable de estimular la maduración de los MKs y de la liberación de plaquetas. Esta hormona se genera principalmente en el riñón y parcialmente en el hígado y en el bazo [350]. Cada MK es capaz de dar lugar a 10-30 proplaquetas que, una vez maduras, pueden liberar de 1000 a 3000 plaquetas [351].

La producción de plaquetas a partir de las unidades formadoras de MKs (BFU-MEG) ocurre en cuatro estadíos (Fig. 10).

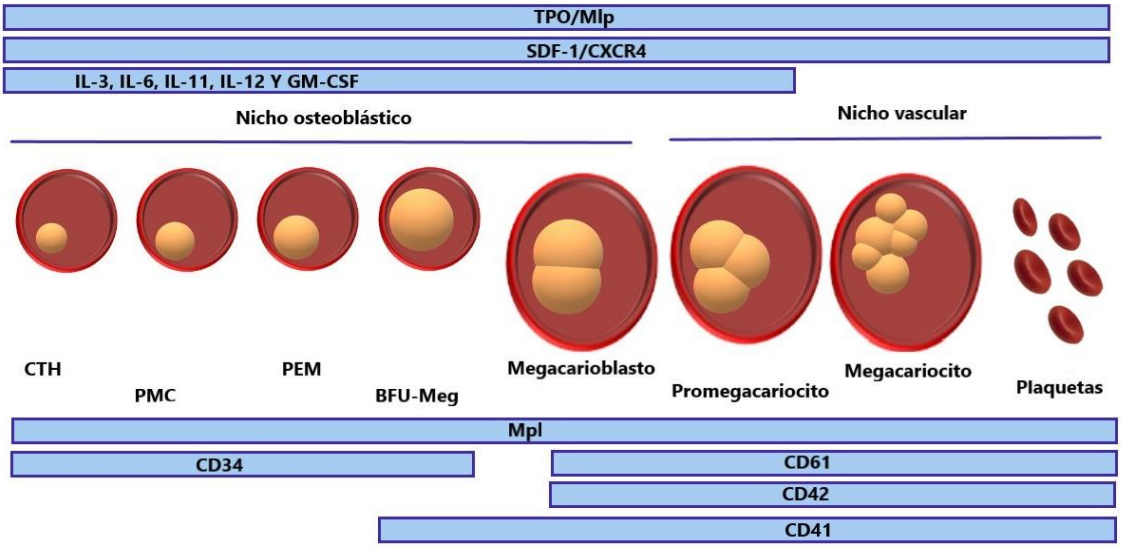


Figura 10. Proceso de maduración y diferenciación de los MKs. La imagen indica cada uno de los estadios que tienen lugar en la producción de las plaquetas y donde ocurre cada uno de ellos.

- Megacarioblasto: es la célula de transición entre el precursor y las células morfológicamente reconocibles. Son las más inmaduras con un diámetro de 10-15 µm, mononucleares y con múltiples nucléolos. Expresan marcadores específicos.

- Promegacariocito o MK basófilo: el megacarioblasto maduro se convierte en una gran célula de 20 µm con ribosomas y citoplasma abundante. Cuenta con un núcleo multilobulado.

- MK granular: sigue aumentando de tamaño (unos 50 µm) y representa un 55% de la población megacariocítica en la médula. Cuenta con un núcleo multilobulado y muestra evidencia de fragmentos citoplasmáticos que contienen membranas, citotúbulos y varios receptores glicoproteicos.

- MK maduro: son las células más grandes (80 µm-150 µm) de la médula ósea. Se someten al proceso de endomitosis. Cuenta con gránulos citoplasmáticos y mitocondrias. El último paso es el proceso de trombopoyesis, gracias al cual se liberan las plaquetas.

El proceso de megacariopoyesis se lleva a cabo principalmente en los sinusoides de la médula ósea, aunque estudios han demostrado que los MKs pueden migrar a través del torrente

sanguíneo hasta los pulmones, donde se acumulan en los vasos, pudiendo ocurrir la biogénesis plaquetaria si fuese necesaria [352-354].

Así pues, la biogénesis plaquetaria en el pulmón puede ocurrir en dos compartimentos distintos [354]. Los MKs migran desde la médula ósea o el bazo hasta la vasculatura pulmonar, donde quedan retenidos hasta que terminan de madurar y liberan a las plaquetas (Fig. 11a). Por otro lado, existen progenitores hematopoyéticos y MKs en el intersticio pulmonar, los que en situaciones de trombocitopenia, migran y restauran las deficiencias hematopoyéticas de la médula ósea (Fig. 11b).

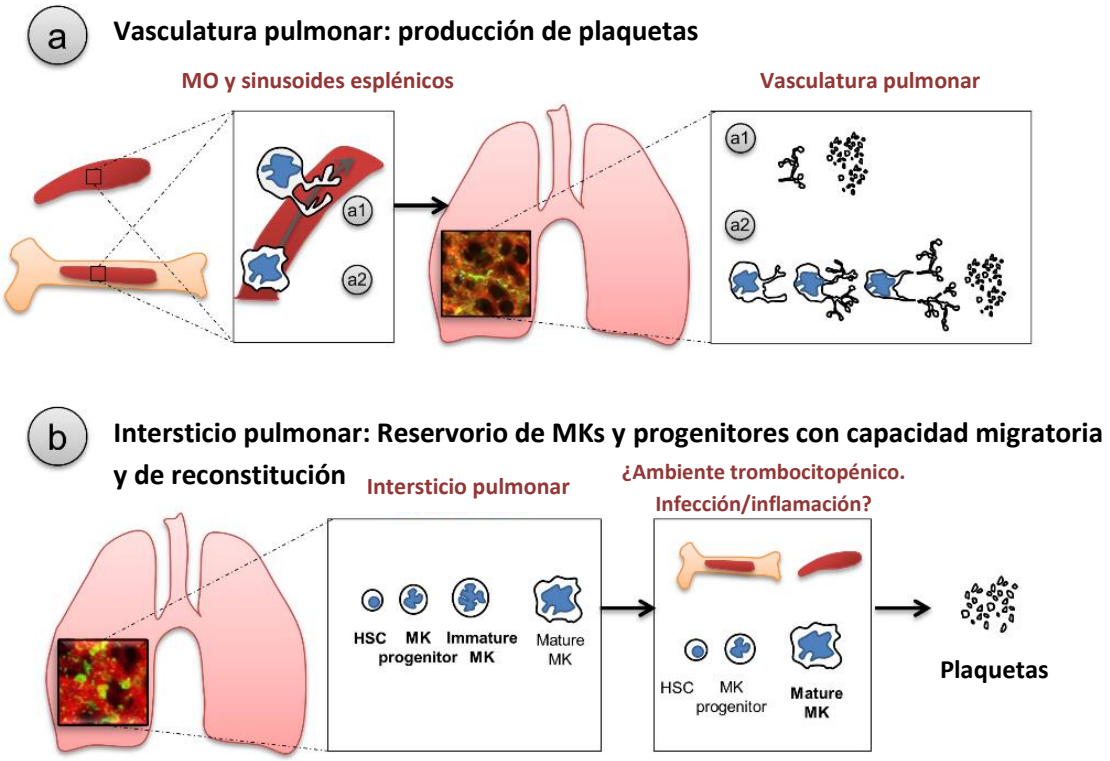


Figura 11. El papel del pulmón en la biogénesis de plaquetas. A) Después de la liberación de las plaquetas (a1) y de los MKs (a2) desde la médula ósea o desde el bazo, éstas son retenidas en la vasculatura pulmonar, donde tiene lugar la formación y expansión de las proplaquetas, así como la liberación de las plaquetas. B) Los MKs maduros e inmaduros, junto con los progenitores hematopoyéticos, son almacenados en el intersticio pulmonar, del cual migran y restauran las deficiencias hematopoyéticas de la médula ósea en situaciones trombocitopénicas. Adaptado de *Lefrancais et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. Nature. 2017; 544 (7648): 105-109.*

1.2.3 REGULACIÓN DE LA MEGACARIOPOYESIS MEDIADA POR TPO

La regulación de la megacariopoyesis, en una situación normal en la que no existe patología alguna, está controlada por los niveles de TPO en médula ósea y en sangre, que son inversamente proporcionales al número de plaquetas. Las plaquetas tienen receptores para la TPO (MpL1), de modo que al aumentar el número de plaquetas en circulación, también aumenta el número de receptores para TPO que la unen e internalizan y, por tanto, disminuyen los niveles de TPO en plasma.

La TPO es una glicoproteína de 30 kDa, que es sintetizada principalmente y de forma constitutiva por los hepatocitos del hígado, especialmente por células endoteliales de los sinusoides hepáticos y células del parénquima hepático, aunque también se produce en los riñones y en el estroma de la médula ósea. Hay estudios que demuestran la existencia de ARN mensajero para la síntesis de TPO en células del músculo liso, cerebro, ovario, testículo y pulmón, por lo que se piensa que estos tejidos también tienen alguna función en la megacariopoyesis [346, 355].

El Mpl, miembro de la superfamilia del receptor de citoquinas hematopoyéticas, se encuentra en la superficie de plaquetas circulantes, en MKs, en células madre hematopoyéticas (HSC) y en hemangioblastos.

Aunque durante años se ha identificado a la TPO como el principal regulador en la producción de plaquetas, se ha demostrado que la maduración de los MKs y la producción de plaquetas puede ser un proceso independiente de TPO [356]. En procesos inflamatorios y en condiciones citopénicas, se produce un incremento de los niveles séricos de varias citoquinas proinflamatorias, entre ellas la IL-1α. Esta citoquina es un factor que produce la ruptura de la membrana del MK maduro, dando lugar a la liberación de las plaquetas [357].

1.2.4 ESTRUCTURA PLAQUETARIA

Las plaquetas circulan por el torrente sanguíneo en estado inactivo, manteniendo una forma discoide, gracias a las prostaglandinas, al óxido nítrico y ADPasas del endotelio vascular. Una vez que las plaquetas se activan, cambian su conformación, emiten pseudópodos, se adhieren y se agregan entre sí o con otros tipos celulares.

Las plaquetas tienen un citoesqueleto muy organizado y un sistema de receptores de membrana que condicionan su alta reactividad. En su interior presentan distintos tipos de gránulos que contienen diferentes moléculas y que son secretadas en caso de activación (Fig. 12).

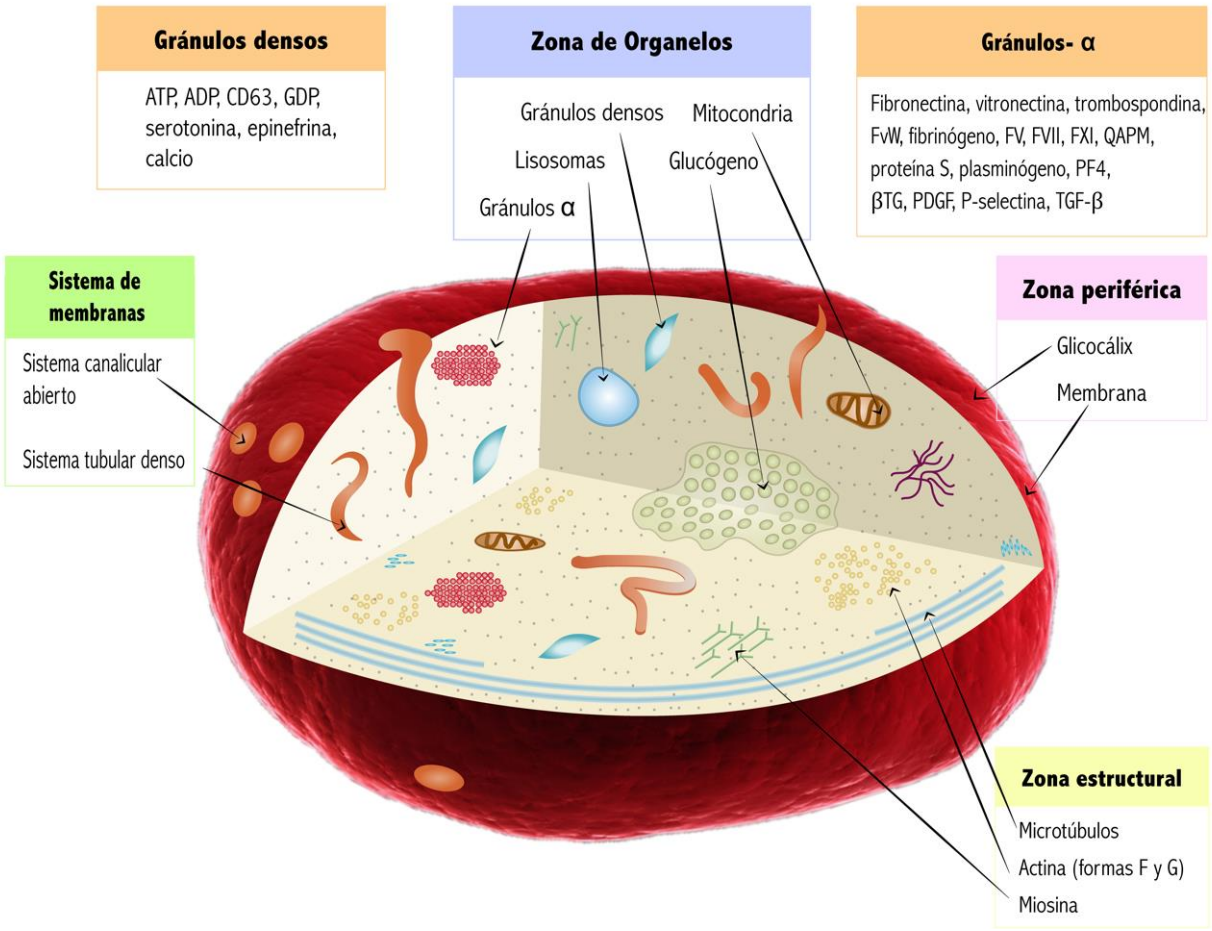


Figura 12. Estructura interna de la plaqueta.

Membrana plasmática de la plaqueta

La membrana plasmática plaquetaria se extiende por diversas ramificaciones del sistema canalicular conectado a la superficie (SCCS).

Interviene activamente en la hemostasia ya que cuenta con receptores específicos y además, durante la agregación plaquetaria proporciona una superficie esencial para que se produzca la coagulación.

La membrana plaquetaria cuenta con tres capas, siendo la más externa el glicocálix, una bicapa lipídica intermedia y, el área submembranosa, la más interna.

- El glicocálix

El glicocálix constituye la cubierta externa plaquetaria donde se encuentran las glicoproteínas de membrana (GPs). La mayor parte de las GPs pertenecen a la familia de las integrinas y participan

en la adhesión y agregación plaquetaria. En el glicocálix se encuentran situados receptores que responden a distintos estímulos y transmiten señales a los orgánulos internos y que se comentarán posteriormente.

- Bicapa lipídica

En esta bicapa lipídica, los lípidos más abundantes son los fosfolípidos que forman la estructura básica de la plaqueta. También existen lípidos neutros (triglicéridos, colesterol y ácidos grasos libres), distribuidos asimétricamente entre los fosfolípidos.

La distribución de los fosfolípidos tiene gran importancia en la hemostasia. Cabe destacar la presencia de fosfatidilinositol, que participa en la activación plaquetaria al proporcionar ácido araquidónico que se convierte en tromboxano A_2 (TXA_2) durante el proceso; además hidroliza a la fosfolipasa c, generando intermediarios que promueven la activación plaquetaria, exponiendo PS en la membrana plaquetaria, lo que promueve la coagulación al favorecer el ensamblaje del complejo FVIII-FIX y FX-FV.

- Área submembranosa

Está situada por debajo de la bicapa lipídica de la membrana plaquetaria y es el punto donde confluyen los dominios citoplasmáticos de las GPs de membrana, para interaccionar con distintas proteínas para la transmisión de señales. También se puede encontrar calmodulina y fibras de miosina y actina del citoesqueleto plaquetario, esencial para el mantenimiento de la estructura de la plaqueta y para producir los cambios conformacionales necesarios para su activación.

Glicoproteínas implicadas en hemostasia

- **GPIIb-IIIa (CD41-CD61, αIIbβ3):** subunidades del receptor de fibrinógeno, presentes también en la membrana de los gránulos-α y en MKs. Este receptor, además, puede unir FvW, fibronectina, trombospondina y vitronectina. Participa en la activación, adhesión e interacción plaqueta-plaqueta durante la formación del coágulo. La activación plaquetaria se produce cuando agonistas como el ADP, la trombina, el colágeno o la epinefrina se unen a sus receptores presentes en la membrana de las plaquetas y desencadenan una señalización intracelular (aumento en la concentración de calcio intracelular, fosforilación de proteínas) que induce una señalización de adentro hacia afuera (“inside-out”), que provoca un cambio conformacional en el receptor [358] que, de este modo, aumenta su afinidad por el fibrinógeno, FvW o fibronectina. Una vez unidos los ligandos de adhesión

al receptor, se produce una señalización de afuera hacia dentro ("outside-in"), induciendo así la agrupación de diversas integrinas y la adhesión plaquetaria (Fig. 13).

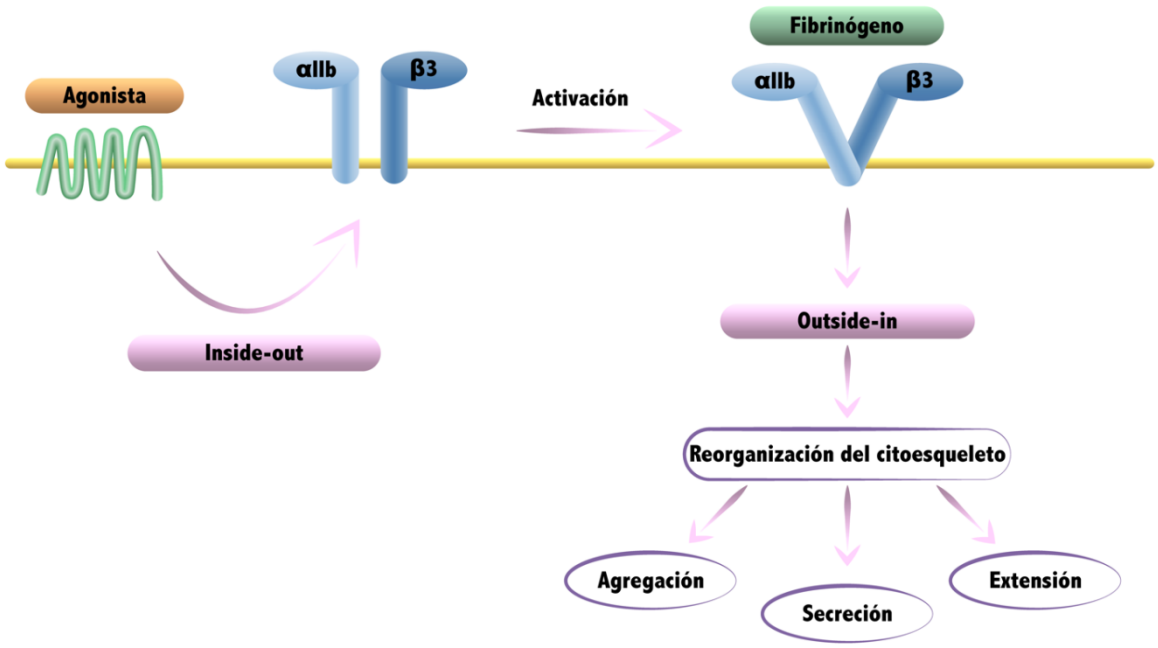


Figura 13. Cambios conformacionales del receptor de fibrinógeno. La interacción de agonistas con sus receptores provoca una señal inside-out que cambia la conformación del receptor del fibrinógeno para aumentar su afinidad por el ligando. Tras esta unión se generan señales del exterior al interior de la plaqueta (señal outside-in), que activan distintos procesos en cascada, provocando la agregación y la adhesión plaquetaria, así como en la secreción de sustancias proactivas.

- **Receptor GPIb-IX-V (GPIb):** se trata de un complejo multimérico formado por GPIbα, GPIbβ, GPIX y GPV. Es el receptor para el FvW que media la adhesión de la plaqueta al subendotelio, y que actúa como puente entre el colágeno expuesto debido al daño [359]. Tras la activación plaquetaria, las calpaínas también se activan, y se produce una reducción en la expresión del complejo GPIb-IX-V, además de la liberación de las subunidades GPIb y GPV. Así se mejora la actividad procoagulante de las plaquetas y, en consecuencia, se favorece la producción de la trombina [360] y de la fibrina [361].

- **GPIa-IIa (CD49b-CD29):** se trata de la integrina α2β1 que funciona como receptor del colágeno y que participa en la adhesión de las plaquetas al colágeno de la matriz subendotelial [362].

- **GPVI:** receptor del colágeno [363], aunque también puede ser activado por fibrina, laminina, histonas y adinopectina [364], participando en la formación del trombo.

- **GPIV (CD36):** presente en la superficie de plaquetas, monocitos, células endoteliales y eritrocitos. Actúa como receptor del colágeno tipo II y también une trombospondina, ácidos grasos y lipoproteínas oxidadas, y participa en la transducción de señales. Se ha detectado en la superficie plaquetaria, en el sistema canalicular y en la membrana de gránulos-α [365].

- **P-selectina (CD62P):** pertenece a la familia de las selectinas. Se encuentra en los gránulos de Weibel-Palade de células endoteliales y en los gránulos-α de las plaquetas, expresándose en la superficie cuando la plaqueta se activa. Media la interacción entre las plaquetas y los leucocitos mediante la interacción de la P-selectina plaquetaria con su receptor, la P-selectina glicoproteína-1 (PSGL-1), presente en leucocitos. Además permite la interacción entre las plaquetas y el endotelio y que los agregados plaquetarios se estabilicen [366].

La tabla 12 muestra los receptores de la membrana plaquetaria acoplados a proteínas G que están implicados en la hemostasia.

Receptor	Ligando	Proteína G
PAR1	Trombina	Acoplado a proteína G_1 reduce cAMP; acoplado a G_q y G_{12} incrementa IP3 y DAG.
PAR4	Trombina	Acoplado a las proteínas G_q y G12 que incrementa IP3 y DAG.
$P2Y_1$	ADP	Acoplado a la proteína G_q que incrementa DAG e PI3.
$P2Y_{12}$	ADP	Acoplado a proteína G_1 que incrementa cAMP.
TPα y TPβ	TXA_2	Acoplado a proteína G_q que incrementa DAG e IP3.
α_2-adrenérgico	Epinefrina	Acoplado a proteína G_1 reduce cAMP, potencia los efectos de ADP, trombina y TXA_2.
IP	PGI_2	Proteína acoplada a Gs que incrementa cAMP para inhibir la activación.

Tabla 12. Receptores de membrana acoplados a proteínas G presentes en las plaquetas.

Elementos del citoesqueleto

1. Sistemas membranosos

- **Sistema canalicular abierto:** La membrana plasmática invade el interior de la plaqueta y se forma un único sistema canalicular conectado a la superficie, conocido como sistema canalicular abierto [367]. Este sistema consiste en una serie de invaginaciones tubulares que actúa como una vía tanto para la entrada de elementos externos al interior de la plaqueta como para la liberación del contenido de los gránulos hacia el exterior [368, 369]. También representa un reservorio interno de membrana que se usa en la formación de los pseudópodos durante la activación de las plaquetas, momento en que se produce un aumento de la superficie de la membrana, sin que ésta se sintetice *de novo* [370].

- **Sistema tubular denso:** El sistema tubular denso es un remanente del retículo endoplasmático rugoso del MK del que procede la plaqueta y se distribuye por todo el citoplasma plaquetario [371]. Entre sus funciones se encuentra la captación de iones de calcio (Ca^{2+}) y la liberación de éstos cuando las plaquetas son activadas. También almacena las enzimas que llevan a cabo la activación plaquetaria, incluida la fosfolipasa A, la ciclooxigenasa, el Ca^{2+}, las ATPasas y la tromboxano sintetasa.

Estructuras inespecíficas de las plaquetas

Estas estructuras se refieren a ciertos elementos que se encuentran en la plaqueta y que son comunes a otros tipos celulares. Entre ellas se encuentran los lisosomas, que contienen hidrolasas ácidas involucradas en la degradación de lípidos, proteínas y carbohidratos [372] y que pueden digerir los componentes de la matriz de los vasos sanguíneos durante la agregación, así como restos de la autofagia; algunas mitocondrias, implicadas en el metabolismo; vesículas revestidas que actúan como mecanismo alternativo a la endocitosis mediada por receptores; los peroxisomas, que contribuyen al metabolismo de lípidos en la plaqueta, ribosomas y RNA mensajero (RNAm).

Orgánulos plaquetarios

- **Gránulos-α**: son gránulos redondos u ovoides con un diámetro entre 200 y 500 nm**.** Hay unos 50-80 gránulos-α por plaqueta y se forman durante la maduración temprana de los

MKs. La matriz puede dividirse en tres zonas: A) zona nucleoide oscura con proteoglicanos y proteínas plaquetarias como β-trombomodulina y factor 4 plaquetario (PF4), B) la zona clara con estructuras tubulares, espaciadas y alineadas, donde se encuentra el FvW en forma multimérica de alto peso molecular y C) una zona intermedia asociada con el fibrinógeno, la trombospondina, la albúmina y factores de crecimiento. En el interior de estos gránulos hay proteínas, algunas endocitadas y otras sintetizadas en el MK. Cuando la plaqueta se activa, la membrana de estos gránulos se fusiona con el SCCS, lo que produce que su contenido salga al exterior donde participan en la adhesión y agregación plaquetaria y colaboran en la coagulación [373].

- **Cuerpos multivesiculares:** son estructuras granulares que contienen distintas vesículas y similares en tamaño a los gránulos-α.

- **Gránulos densos:** hay de 2 a 7 gránulos densos por plaqueta, de 20-30 nm de diámetro. En ellos se almacenan pequeñas moléculas endocitadas. Tienen transportadores de membrana y almacenan calcio y magnesio, ADP, polifosfatos y serotonina (5-HT) [374]. La membrana de estos gránulos contiene GPs que también se encuentran en la membrana plasmática y en la membrana de los gránulos-α o lisosomas, incluyendo CD36 (GP53-granulolisina), P-selectina, GPIIb/IIIa y GPIb/IX [375].

  Después de la activación, el contenido de los gránulos densos es secretado por fusión con la membrana plasmática y las proteínas de membrana son traslocadas a la superficie. La liberación del contenido de estos gránulos es un mecanismo de retroalimentación positiva para la agregación plaquetaria, ya que el ADP es un agonista potente y la serotonina uno débil.

- **Gránulos T:** fueron descubiertos hace relativamente poco. Contienen PDI, VAMP-8 y TLR9 y están relacionados con la respuesta inmune innata y adaptativa durante la inflamación infecciosa y la enfermedad aterosclerótica vascular [376].

1.2.5 REGULACIÓN DEL RECUENTO PLAQUETARIO

Como en todo proceso fisiológico, la producción de plaquetas debe estar en equilibrio con la destrucción de éstas, con el fin de mantener un equilibrio en el recuento plaquetario y evitar que se produzcan hemorragias o trombos en el organismo.

Existen distintos mecanismos por el cual las plaquetas son eliminadas de la circulación, incluyendo el aclaramiento mediado por anticuerpos y la exposición de PS en la superficie, entre otros.

Otro de los mecanismos que tiene lugar en la regulación del recuento plaquetario se produce a través del cambio en la composición de los glicanos de la superficie plaquetaria. La pérdida de parte del ácido siálico de las GPs de la superficie de las plaquetas senescentes por acción de las enzimas sialidasas, enzimas también conocidas como neuroaminidasas, las cuales pueden provenir del compartimento granular de la propia plaqueta [377] o ser extracelulares. Las neuraminidasas liberan el ácido siálico terminal de los glicanos de la superficie plaquetaria dejando expuestos residuos de β-galactosa. La β-galactosa expuesta de los glicanos de estas plaquetas desializadas es reconocida por los receptores Ashwell-Morell del hígado, produciendo el aclaramiento de las plaquetas de la circulación [378].

Otro proceso de aclaramiento plaquetario se produce a través de la muerte programada de éstas, proceso conocido como apoptosis.

Apoptosis plaquetaria

En 1972, Kerr *y cols.* acuñaron el término apoptosis [379] para describir el mecanismo de muerte celular programada, en el cual se producía una contracción citoplasmática, la generación espontánea de burbujas celulares (*blebbing*), la fragmentación nuclear, la fragmentación del DNA y la exposición de PS en el exterior de la membrana plasmática celular, atrayendo a los macrófagos para su ingesta y eliminación [380].

Actualmente se conoce que la apoptosis es un proceso celular clave que funciona como mecanismo de defensa ante reacciones inmunes, cuando las células sufren daño por alguna enfermedad o por agentes nocivos, así como un mecanismo hemostático para mantener las poblaciones celulares en los tejidos [381]. Se ha visto que una apoptosis inadecuada está implicada en diversas enfermedades humanas, incluyendo enfermedades neurodegenerativas como la enfermedad de Huntington [382] y en Alzheimer [383], en daño isquémico [384], en desórdenes autoinmunes [385, 386] y en varias formas de cáncer [387].

Desde su descubrimiento, la apoptosis siempre fue asociada a las células nucleadas. Sin embargo, desde hace algo más de una década, se ha visto que es un proceso que también ocurre en citoplastos enucleados [388] y en plaquetas [389, 390], tanto *in vivo* como *in vitro*.

El mecanismo de apoptosis se produce gracias a la acción de una familia de proteasas, denominadas caspasas. Las caspasas son sintetizadas como proenzimas inactivas en la mayor parte de las células y, una vez activadas, pueden a su vez activar a otras procaspasas, dando lugar a una cascada proteolítica, que amplifica la señal de la ruta apoptótica y que culmina en una rápida muerte celular.

Las caspasas son proteínas altamente conservadas a lo largo de la evolución. Actualmente se conocen 12 caspasas humanas, que se clasifican según si actúan en la apoptosis (-2, -3, -6, -7,- 8,-9, -10 y -12) o en la inflamación (-1,-4,-5,-11). Las caspasas involucradas en la apoptosis, a su vez, se subclasifican en caspasas iniciadoras (-2,-8, -9 y -10) o ejecutoras (-3, -6 y -7) [391, 392]. Estas últimas son activadas una vez que son procesadas proteolíticamente por las caspasas iniciadoras [393].

Existen dos rutas apoptóticas: la vía intrínseca y la vía extrínseca. En plaquetas, la vía intrínseca ha sido más caracterizada que la vía extrínseca.

1. Vía extrínseca

La apoptosis mediante la vía extrínseca involucra la actuación de ligandos de muerte, pertenecientes a la superfamilia del TNF, con los receptores de TNF situados en la superficie celular. Los miembros de esta familia comparten dominios extracelulares de unos 80 aminoácidos llamados “dominio de muerte”, ya que son capaces de inducir apoptosis al ser activados por la unión de su ligando correspondiente. Además, participan en la transmisión de señales desde la superficie celular hasta el interior de las mismas. Las vías de señalización más caracterizadas hasta el momento son las que ocurren a través del ligando Fas (FasL) y su receptor (FasR), así como a través del ligando TNF-α y su receptor TNRF-1.

En el caso de la unión de FasL, se produce la trimerización de Fas. La región citoplasmática del receptor contiene un dominio de muerte que recluta a la proteína adaptadora Fadd (*fas-associating protein with death domain*), la cuál es capaz de interactuar y reclutar a las procaspasas -8 y -10. Cuando la procaspasa-8 se asocia con Fas/Fadd, se forma el complejo de señalización de muerte (DISC- *death inducting signaling complex*). Esto permite que la procaspasa-8 se procese proteolíticamente dando lugar a la caspasa-8 activada, la cual procesa a las procaspasas -3,-6 y -7, con el fin de llevar a cabo el mecanismo de apoptosis.

La proteína adaptadora que interacciona con la región citoplasmática de TNRF-1 se llama Tradd (TNF-*receptor associated death domain*). Tradd recluta a Fadd y, como se ha comentado

anteriormente, Fadd se asocia a la procaspasa-8, formándose el complejo DISC, lo que da lugar a la activación autocatalítica de la procaspasa-8, iniciándose el mecanismo apoptótico (Fig. 14a).

2. Vía intrínseca

La apoptosis que tiene lugar a través de la vía intrínseca es una ruta dependiente de mitocondrias y puede estar desencadenada por estímulos no mediados por receptores, tales como citoquinas, daño intracelular, oncogenes o exposición a agentes quimioterapéuticos [394].

En la apoptosis por vía intrínseca destaca la función de las proteínas de la superfamilia Bcl-2, que se subdividen en tres grupos: proteínas antiapoptóticas (Bcl-2, Bcl-x_L, Mcl-1, Bcl-w y A1), la familia de proteínas proapoptóticas multidominio (Bax y Bak) y las proteínas proapoptóticas tipo BH3-only (Bid, Bim, Puma, Noxa, Bad, BCL-Xs, Bik, Hrk, Bmf). Las proteínas antiapoptóticas restringen la actividad de las proteínas Bak y Bax, que son las encargadas de permitir la liberación del citocromo c tras el daño mitocondrial, mientras que las proteínas tipo BH3-only, pueden inhibir a las proteínas antiapoptóticas y transmitir las señales pro-muerte de Bak y Bax [395].

En una situación normal, las proteínas antiapoptóticas controlan la actividad de las proteínas proapoptóticas. La actividad de estas proteínas puede verse alterada por distintos agentes físico-químicos, induciendo la permeabilidad de la membrana mitocondrial externa (MOMP) y permitiendo la salida de ciertas proteínas, como el citocromo c, desde el espacio intermembranoso hasta el citosol.

El citocromo c es un factor proapoptótico que, una vez en el citosol, desencadena la oligomerización del factor apoptótico 1 activador de proteasas (Apaf-1), proteína con un dominio CARD que le permite interactuar y reclutar a la procaspasa-9 hasta el complejo proteico conocido como apoptosoma. La formación de un complejo multimérico entre el citocromo c y Apaf-1 permite la activación, mediante autocatálisis, de la procaspasa-9. Una vez activada, la caspasa-9 se libera del complejo y activa a las caspasas efectoras -3 y -7, las cuales rompen una gran cantidad de sustratos intracelulares que producen daño en el DNA, suprimen la transcripción y traducción de proteínas, e impiden llevar a cabo procesos celulares esenciales.

En mamíferos se han encontrado cuatro genes que codifican para proteínas inhibidoras de caspasas (IAP – *inhibitory of apoptosis proteins*), como Xiap, c-Iap-1, c-Iap-2, que se pueden unir a las caspasas -3,-7 y a la procaspasa-9, inactivándolas. De forma paralela a la salida del citocromo c de la mitocondria, y constituyendo un segundo punto de control de la apoptosis, también se

produce la liberación de la proteína SMAC/DIABLO desde la mitocondria, que inhibe a los IAPs uniéndose a ellos y promoviendo así la activación de las caspasas.

La vía intrínseca de la apoptosis se puede conectar con la vía extrínseca, puesto que una vez que la caspasa-8 se activa por lo receptores de la vía extrínseca, esta caspasa activa a la proteína Bid, provocando la apertura del poro mitocondrial y la activación de la caspasa-9 (Fig. 14b).

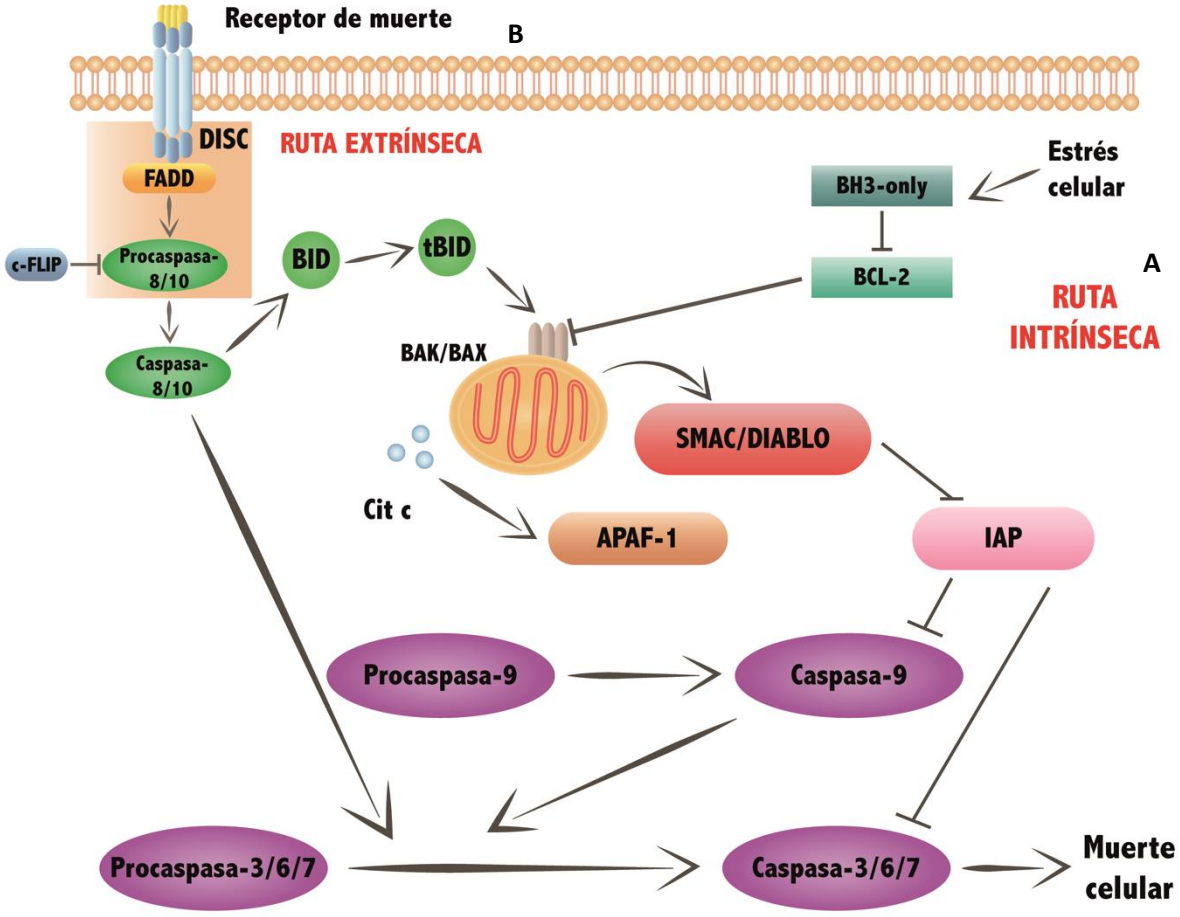


Figura 14. Las rutas apoptóticas. Hay dos vías de apoptosis, la intrínseca y la extrínseca. A) La vía intrínseca es activada por estrés celular. Cuando se activan, las proteínas proapoptóticas only-BH3 inhiben las proteínas antiapoptóticas BCL-2 y se induce la oligomerización BAK/BAX, la permeabilización de las mitocondrias y la liberación de citocromo c (cit c) y SMAC/DIABLO. El citocromo c forma un complejo con caspasa-9 y con APAF-1, lo que lleva a la activación de la caspasa-9. La caspasa-9 activa las caspasas ejecutoras (caspasa-3, -6 y -7) e induce la muerte celular. B) La vía extrínseca se activa mediante señales de muerte mediadas por ligandos de muerte. El ligando de muerte activa el receptor de muerte, que forma el complejo DISC y envía una señal para activar a las caspasas-8 y -10, las cuales activan a la caspasa-3. Adaptado de Shadia Zaman et al. *Targeting the apoptosis pathway in hematologic malignancies*. Leuk Lymphoma. 2014; 55(9): 1980–1992.

1.2.6 FORMACIÓN DE MPs

Las MPs se definen como pequeñas vesículas de 0,1-1 µm de diámetro que pueden proceder de plaquetas, células endoteliales, leucocitos y eritrocitos como consecuencia de la activación o de la apoptosis celular. Las MPs procedentes de plaquetas son las más abundantes en el torrente sanguíneo (70-90%).

Las MPs no tienen núcleo y expresan en su superficie marcadores característicos de las células de las que proceden. Tienen una distribución asimétrica de los fosfolípidos en su membrana, presentando fosfatidilcolina y esfingomielina en la cara extracelular, y fosfatidiletanolamina y PS en la cara interna. La flipasa, la flopasa y la escramblasa son transportadores de fosfolípidos que se encargan de mantener dicha asimetría. En una situación normal, la flipasa es la única enzima que se encuentra activada, mientras que la flopasa y escramblasa solo se activan en caso de apoptosis o activación.

La formación de MPs ocurre en una serie de pasos. Cuando se produce la apoptosis o la activación plaquetaria, el calcio es liberado desde el retículo endoplasmático, inactivando a la flipasa y, por el contrario, activando a la flopasa y a la escramblasa, induciendo así la pérdida de asimetría de fosfolípidos en la membrana y quedando la PS expuesta en la superficie (Fig. 15).

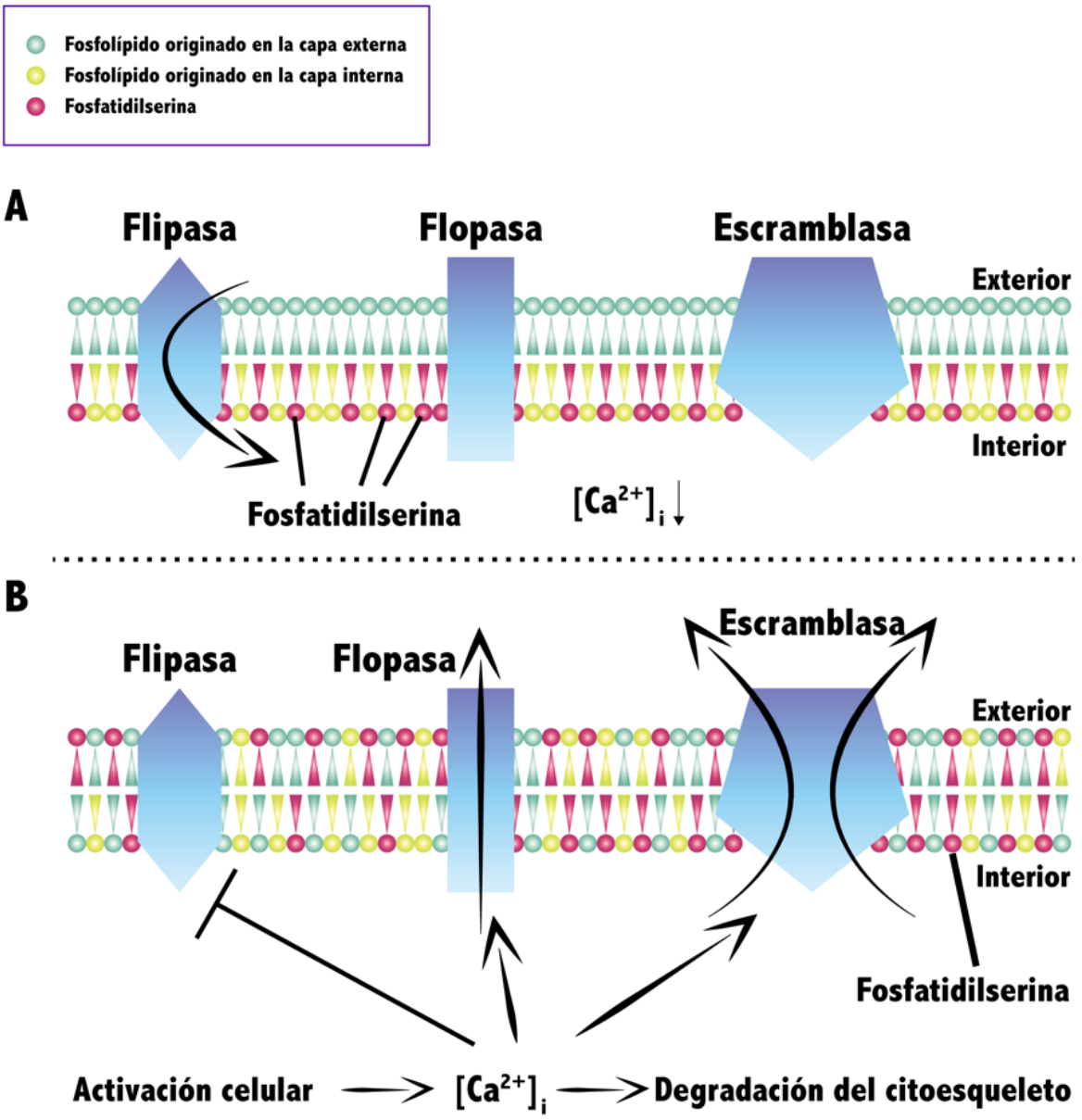


Figura 15. Formación de MPs. A) Cuando la célula se encuentra en reposo, la flipasa se encuentra activada manteniendo la distribución asimétrica de los fosfolípidos de membrana. B) Una vez que la célula se activa, la concentración de Ca^{2+} intracelular aumenta y se activan la flopasa y la escramblasa, perdiendo la asimetría de los fosfolípidos de membrana. Adaptado de Burnier L et al. Thromb Haemost, 2009. 101:439-451.

A continuación, el calcio liberado lleva a la activación de la calpaína y de la gelsolina, siendo esta última exclusiva de las plaquetas. Estas dos enzimas actúan hidrolizando los puentes de actina y, por tanto, disminuyendo la asociación de la actina con las GPs de membrana. De esta forma se generan las vesículas que darán lugar, en último término, a las MPs (Fig. 16).

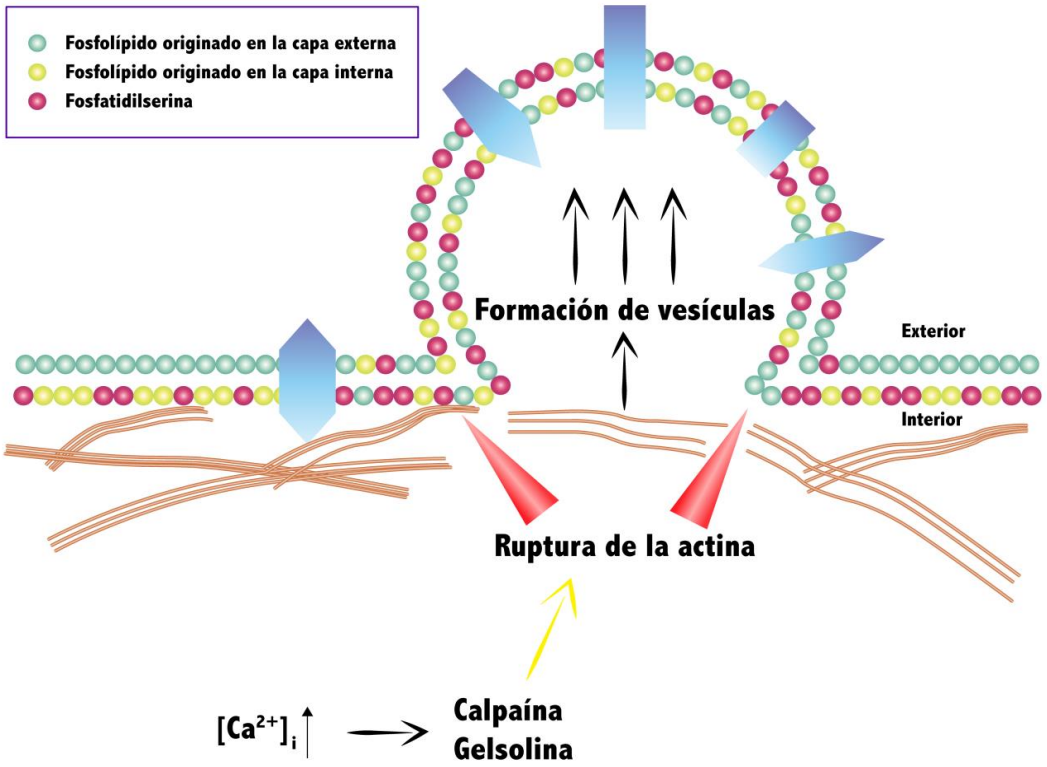


Figura 16. Liberación de MPs. La calpaína y la gelsolina actúan en la etapa final de la formación de MPs, hidrolizando puentes de actina para dar lugar a los ectosomas previos a las MPs. Adaptado de Burnier L et al. Thromb Haemost, 2009. 101:439-451.

Con el paso del tiempo, las MPs procedentes de distintos tipos celulares han sido identificadas como una fuente de FT, capaz de formar complejos con el FVII-FVIIa, entre otros (Fig. 17). Por otro lado, la exposición de PS hace que distintos factores de coagulación se puedan unir y dar inicio a la cascada de coagulación, induciendo la generación de trombina.

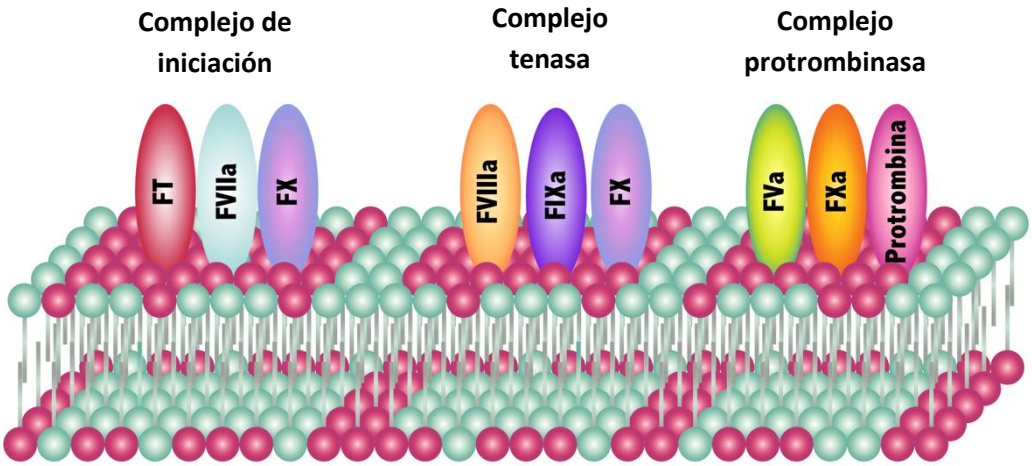


Figura 17. Ensamblaje de los complejos de coagulación en la membrana lipídica. La presencia de fosfolípidos aniónicos, tales como PS (en rojo), facilita la unión de FVIIa, FIXa, FXa y protrombina por interacción con ciertos dominios de las proteínas. Adaptada de Owens *et al.,* Circulation Research. 2011; 108: 1284-97.

Se ha visto que la generación de MPs es un proceso fisiológico y un componente patogénico en muchas enfermedades. Las MPs derivadas de las plaquetas y de tejidos ricas en FT (monocitos y células endoteliales) juegan un importante papel en la hemostasia, ya que son capaces de acelerar la formación de trombina, promoviendo un estado procoagulante. También están involucradas en la inflamación, en la respuesta inmune, en la angiogénesis, en la regeneración tisular y en la metástasis, así como en las comunicaciones célula-célula.

1.3 HEMOSTASIA

La hemostasia se define como el conjunto de mecanismos fisiológicos que permite detener las hemorragias. El objetivo de estos procesos es mantener la fluidez de la sangre dentro de los vasos, detener la pérdida de sangre, así como restaurar la pared vascular.

Los componentes de la hemostasia son el sistema vascular (estructura y estado funcional, así como los factores de la pared vascular), el sistema de coagulación (plaquetas y factores e inhibidores de la coagulación) y el sistema fibrinolítico (factores e inhibidores de la fibrinolisis).

Del mismo modo se distinguen cuatro fases en la hemostasia: 1) la vasoconstricción de la zona lesionada, 2) la formación del agregado plaquetario, 3) la generación de fibrina como último paso de la coagulación y 4) la eliminación de la fibrina formada por el proceso de la fibrinolisis.

1.3.1 Sistema vascular

El sistema vascular está formado por arterias, venas y capilares, cuyas paredes están recubiertas por las células endoteliales que poseen propiedades coagulantes y anticoagulantes. En situaciones normales, donde no existe ninguna lesión, el endotelio impide la coagulación mediante la inhibición de la adhesión plaquetaria (síntesis de prostaciclina de los fosfolípidos de membrana y del factor de relajación derivado del endotelio (EDRF)), de la activación de la PCR (síntesis de trombomodulina), de la inactivación de los factores de coagulación (síntesis de glicoaminoglicanos), de la inhibición de la vía extrínseca de la coagulación (síntesis del inhibidor de la ruta del factor tisular [TFPI]) y potenciando el mecanismo de fibrinolisis (síntesis del activador tisular del plasminógeno [tPA]).

Por el contrario, cuando se produce una lesión vascular, el vaso dañado sufre una contracción mediada por el sistema nervioso simpático de forma que se reduce el flujo sanguíneo por la zona dañada. Además, se activan mecanismos procoagulantes del endotelio favoreciendo la adhesión plaquetaria y leucocitaria (exposición o síntesis de colágeno, fibronectina, FvW, selectinas y factor

activador plaquetario [PAF]), favoreciendo de este modo la vía extrínseca (liberación de FT) e intrínseca de la coagulación (formación de FVa y FVIIIa) e inhibiendo la fibrinolisis (síntesis del inhibidor del activador del plasminógeno [PAI-1]). Las plaquetas que se encuentran en circulación reconocen la lesión gracias a los distintos receptores de la membrana plaquetaria. Esta reducción del flujo circulatorio favorece la adhesión de las plaquetas y de los factores de coagulación de la zona dañada.

1.3.2 Sistema de coagulación

Las funciones del sistema de coagulación incluyen el sellado de la lesión vascular, la formación del tapón plaquetario primario con su posterior refuerzo con fibrina, así como la retracción del coágulo formado.

Una vez que se produce la lesión del endotelio, las plaquetas circulantes interaccionan con el colágeno subendotelial y con el FvW expuestos gracias a los receptores GPVI y GPIb/V/IX, respectivamente. Estas uniones, aunque son débiles, son suficientes para favorecer la síntesis de serotonina y TXA_2, que intervienen en la vasoconstricción, así como la síntesis de ADP que favorece la activación y el reclutamiento de más plaquetas.

Esto también permite que ciertas integrinas, como el α2β1 (receptor del colágeno) y αIIbβ3 (receptor de fibrinógeno, del FvW y de la fibronectina) tengan mayor afinidad por sus ligandos, lo que permite que las plaquetas se adhieran firmemente a la matriz del subendotelio y que se agreguen entre ellas. Todo este proceso se conoce como *hemostasia primaria* (Fig. 18) y da lugar a la formación de un tapón hemostático primario inestable que ha de ser fortalecido mediante la formación de una malla de fibrina a través de la *hemostasia secundaria.*

En la fase de inicio de la coagulación, tras la rotura del endotelio, las células subendoteliales exponen FT en su superficie, que actúa como receptor y cofactor del FVII, convirtiéndolo en FVII activado (FVIIa). Así se forma el complejo FVIIa-FT, que se encarga de catalizar la activación del FX y del FIX, dando lugar a FXa y FIXa.

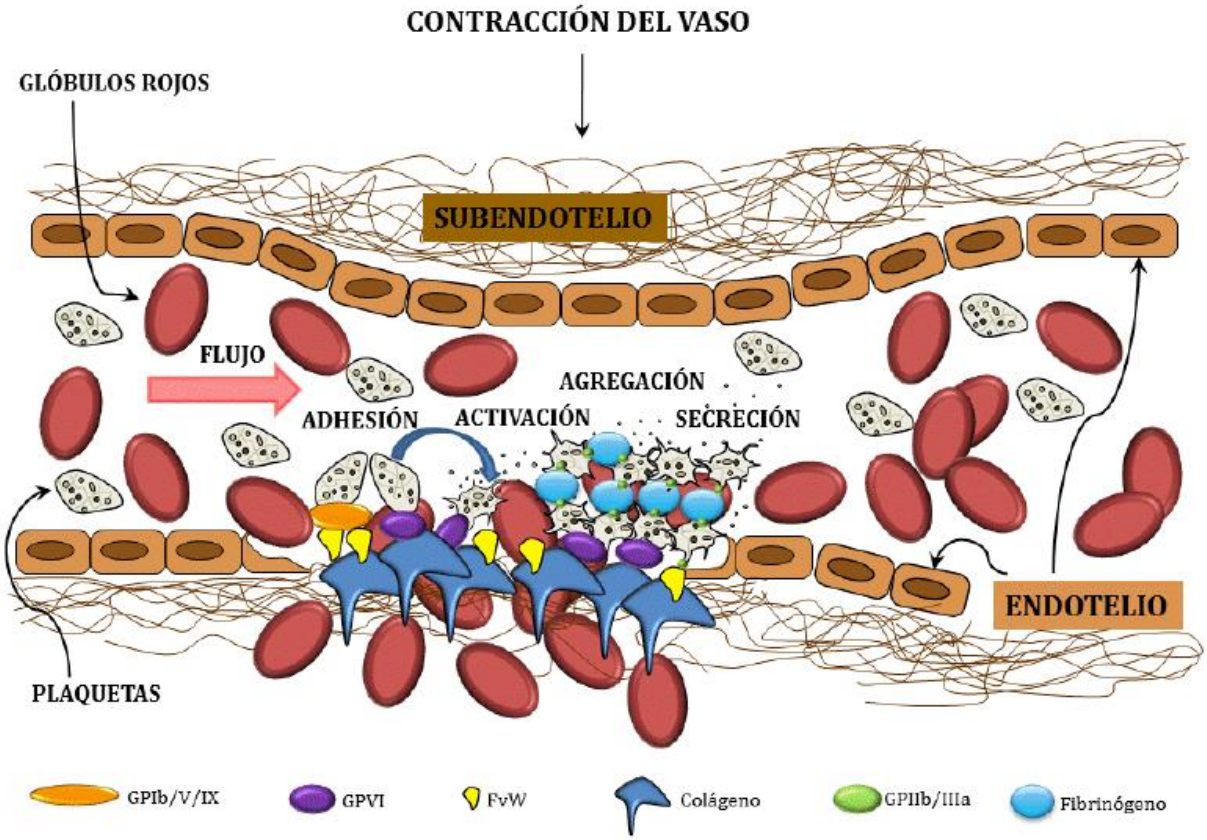


Figura 18. Representación de la hemostasia primaria. Cuando se produce una lesión, tiene lugar un espasmo vascular, tras el cual se produce la adhesión plaquetaria al colágeno y al FvW del subendotelio, la activación de las plaquetas y la agregación de éstas gracias al fibrinógeno plasmático. Así se produce la formación del tapón primario.

Este FIXa que se genera puede difundir hasta las plaquetas activas más cercanas, interaccionando con receptores plaquetarios específicos y con su cofactor, el FVIIIa, de forma que activa al FX directamente sobre la superficie plaquetaria, haciéndolo partícipe de la fase de propagación.

El FXa generado anteriormente se une y activa al FV y junto con el FVa liberado de las plaquetas activadas cercanas, se forma el complejo protrombinasa (FVa-FXa), que actúa sobre la protrombina (FII) dando lugar a la trombina (FIIa), generando así una pequeña cantidad de ésta que no es suficiente para dar lugar a un coágulo estable, siendo necesario aumentar la generación de trombina en la fase de amplificación.

El FXa que no se une para formar el complejo protrombinasa es inhibido por el TFPI a través de un complejo cuaternario (FT-FVIIa-FXa-TFPI) o por la antitrombina (AT), de manera que la formación del coágulo se limita a la zona lesionada y se impide la difusión del FX a otras células (Fig. 19).

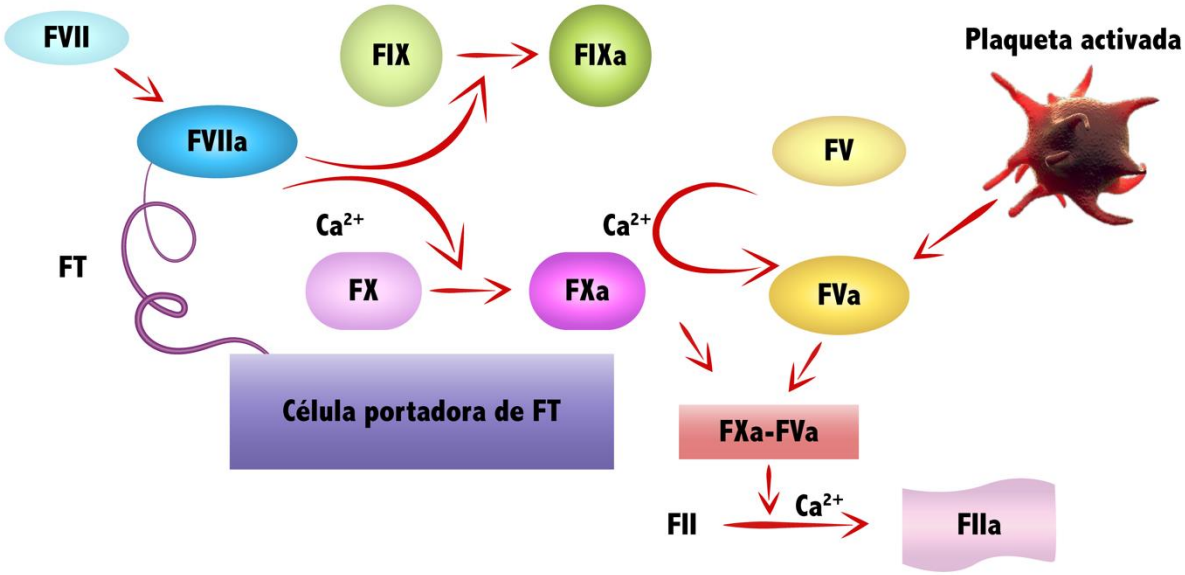


Figura 19. Representación de la fase de inicio en la vía extrínseca de coagulación.

La sangre también puede coagular cuando entra en contacto con una superficie cargada negativamente y distinta del endotelio vascular, dando comienzo a la vía intrínseca de la coagulación. Este contacto hace que el FXII interaccione con la superficie gracias a sus cargas positivas, sufriendo un cambio conformacional y autoactivándose. Esta vía es la que se utiliza *in vitro* para determinar el tiempo de tromboplastina parcial activado (aPTT) y, en consecuencia, la presencia/función de las proteínas de la coagulación que intervienen en la vía intrínseca.

El FXIIa actúa sobre la precalicreína unida a la superficie endotelial que se encuentra formando un complejo con el quininógeno de alto peso molecular (HMWK), produciendo calicreína, la cual también puede activar más cantidad de FXII mediante un proceso conocido como amplificación recíproca.

El FXIIa puede, por un lado, activar a más FXII por autoamplificación y/o activar al FXI que está unido a la superficie en complejo con el HMWK. Tras este paso no se conoce con exactitud el mecanismo que lleva a la generación final de trombina.

Por un lado, se cree que el FXIa actúa sobre el FIX produciendo FIXa. Este FIXa activaría al FX generado pequeñas cantidades de trombina (fase de inicio), aunque parece que el FIX no posee buena actividad catalítica en ausencia de su cofactor, el FVIIa, por lo que se hipotetiza que el FXIa también sería capaz de producir pequeñas cantidades de FVIIIa y FVa. Este FVIIIa formaría un

complejo sobre la superficie plaquetaria con el FIXa conocido como complejo tenasa (FIXa-FVIIIa), que activaría al FX [396]. Este FXa formaría el complejo protrombinasa con el FVa mediante iones de Ca^{2+}, dando lugar al complejo protrombinasa (FXa-FVa), que producirían una cantidad importante de trombina, pasando de la fase de inicio a la fase de amplificación de la coagulación (Fig. 20).

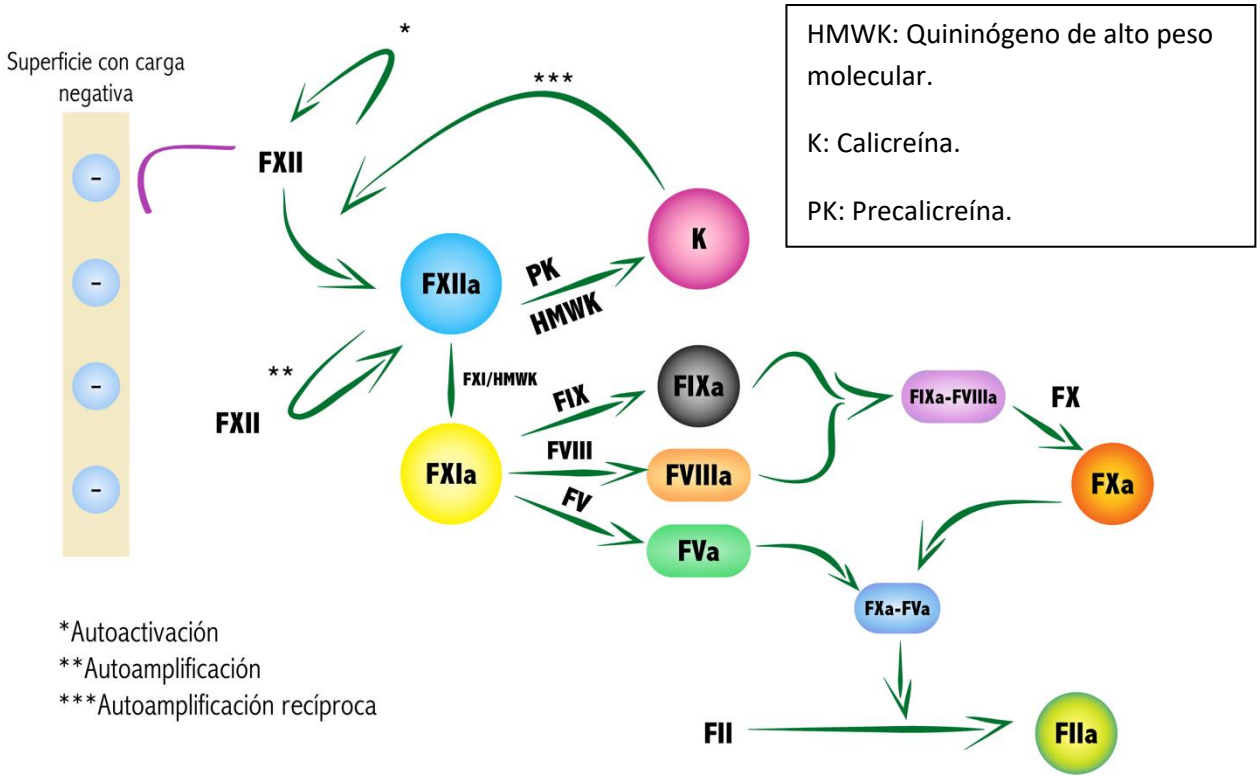


Figura 20. Representación de la vía intrínseca de la coagulación.

En este punto, las vías intrínseca y extrínseca de la coagulación convergen en la vía común.

Las pequeñas cantidades de trombina generadas son responsables de desencadenar la activación de las plaquetas que llegan a la zona de la lesión, así como también de producir la activación del FV, FVII y FXI sobre la superficie plaquetaria, dando paso a la fase de amplificación (Fig. 21). La activación del FVIII hace que el FvW se libere del complejo que se encontraba formando con el FVIII (FVIII-FvW). Este hecho favorece el reclutamiento y la activación de más plaquetas a la zona afectada que, a su vez, proporcionan una superficie procoagulante para la generación de más trombina, dando paso a la fase de propagación.

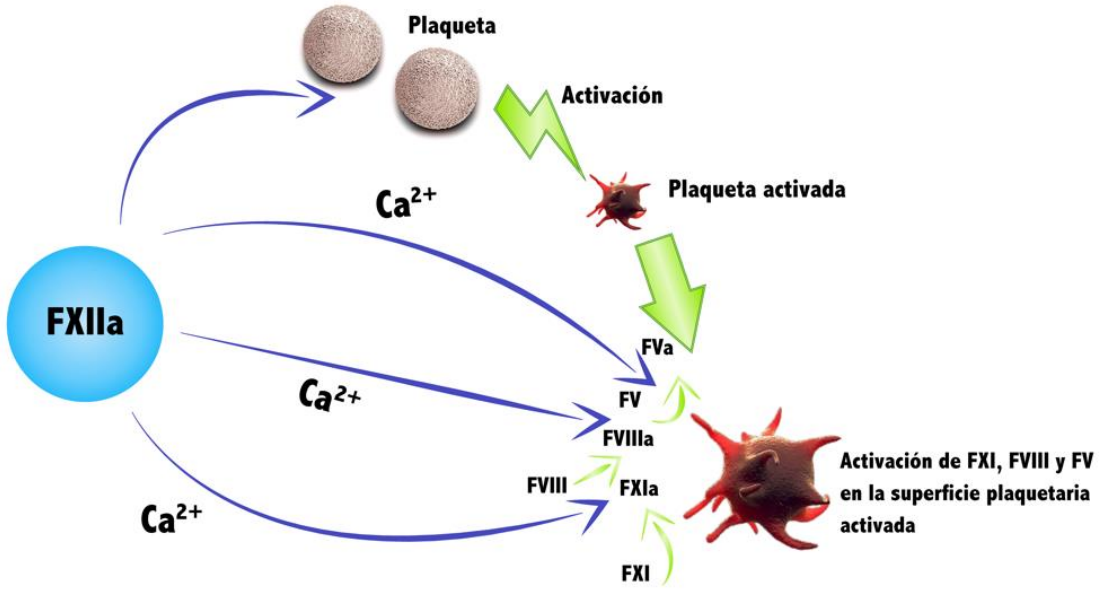


Figura 21. Esquema de la fase de amplificación de la coagulación.

La propagación se produce a partir del FIXa y FVIIIa generado anteriormente, los cuales se asocian a la superficie plaquetaria activada mediante iones de Ca^{2+}, formando el complejo tenasa (FIXa-FVIIIa) que genera FXa, el cual, a su vez, se une al FVa formando el complejo protrombinasa en la superficie de las plaquetas.

El complejo protrombinasa actúa sobre la protrombina originando más trombina que actúa sobre nuevas moléculas de FX, FV, FIX y FVIII activándolos sobre la superficie plaquetaria y cerrando el ciclo de retroalimentación positiva (Fig. 22).

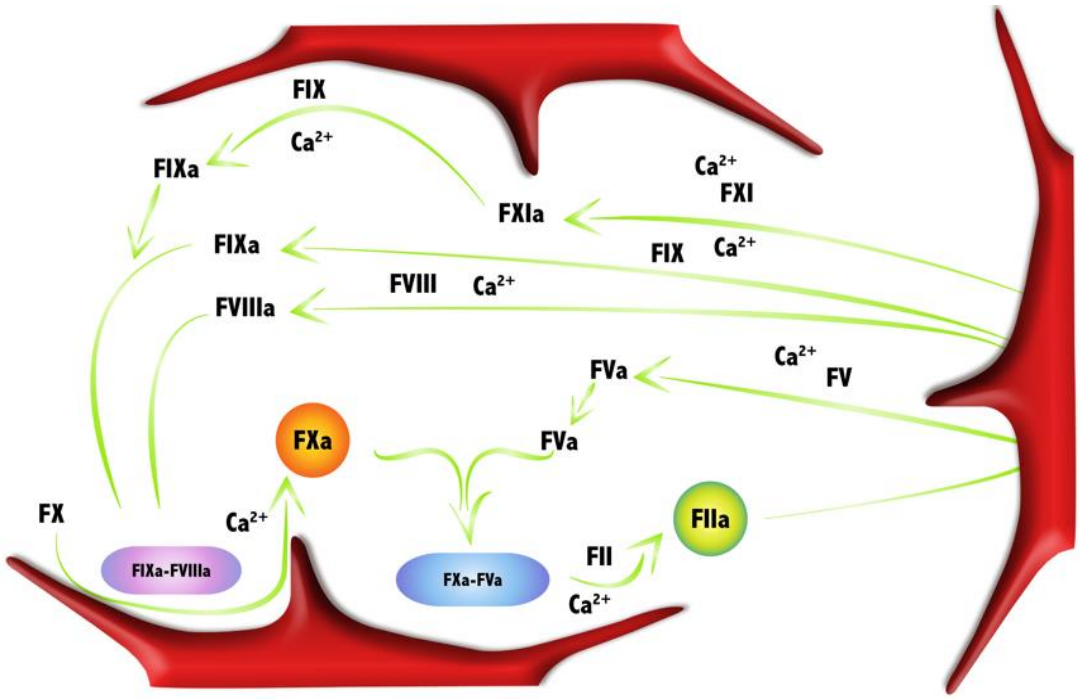


Figura 22. Fase de propagación de la coagulación.

Esta trombina ejerce una actividad proteolítica sobre el fibrinógeno liberando fibrinopéptido A y B. La molécula de fibrinógeno residual, una vez libre de fibrinopéptidos, se conoce como monómero de fibrina. Estos monómeros se unen entre sí mediante enlaces no covalentes [100].

El FXIII, una vez que ha sido activado por trombina [397], une los polímeros de fibrina covalentemente, proporcionando fuerza y estabilidad a la fibrina incorporada en el coágulo que se está formando. Además, la trombina activa al inhibidor de la fibrinolisis activado por la trombina (TAFI), protegiendo al coágulo de su lisis [398].

La retracción del coágulo formado, gracias a la contracción del citoesqueleto plaquetario y la interacción que tiene lugar entre las moléculas de fibrina y el receptor αIIbβ3 de la superficie plaquetaria, contribuyen a la impermeabilización del coágulo y al aumento de la resistencia de éste al flujo sanguíneo, haciéndolo aún más estable.

1.3.3 Sistema fibrinolítico

El sistema fibrinolítico, que debe estar finamente regulado [399], actúa de manera paralela a la cascada de la coagulación para limitar el tamaño del coágulo. También es responsable de la disolución del coágulo una vez que la fibrina ha cumplido su función de manera que se permita la reconstrucción de la superficie tisular. Al igual que la coagulación, la fibrinolisis se puede dividir en tres pasos: la activación del proceso, la formación de fibrina y la descomposición del coágulo.

El principal mediador de la fibrinolisis es la plasmina, que rompe la fibrina en residuos específicos de lisina y arginina, resultando en los productos de degradación de la fibrina, entre los que se encuentra el dímero-D [400], indicador específico de la fibrinolisis [401].

La plasmina es producida por una digestión proteolítica del plasminógeno gracias al tPA y al activador de plasminógeno de tipo uroquinasa (uPA). El tPA y el uPA, liberados del endotelio vascular, son activadores de la fibrinolisis y están regulados por los PAI-1 y PAI-2, los cuales impiden una generación impropia de plasmina mediante una unión irreversible. Por otro lado, la α2-antiplasmina y la α-macroglobulina son glicoproteínas cuya función es inhibir a la plasmina [402] (Fig. 23).

La plasmina, además de degradar a la fibrina, también es capaz de degradar al FVa, FVIIIa, FIXa y al FXIa, así como al fibrinógeno, dando lugar a distintos productos de degradación que interfieren en la polimerización de la fibrina y se unen a las plaquetas produciendo alteraciones funcionales en ellas.

De este modo, el coágulo es eliminado desde la superficie hacia las adheridas al subendotelio hasta que el proceso se completa totalmente [403].

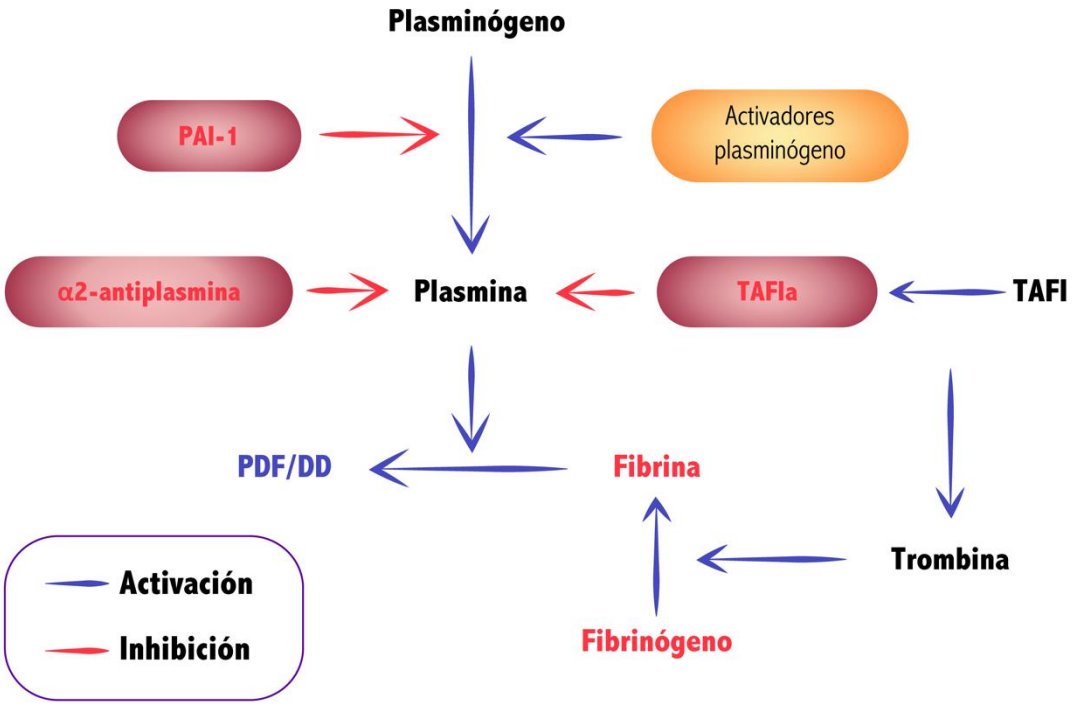


Figura 23. Regulación del sistema fibrinolítico. PDF/DD: producto de degradación del fibrinógeno/dímero D. Adaptada de *Páramo Fernández JA. Sistema hemostático: fisiopatología y aproximación clínica y diagnóstica. Medicine. 2012;11(22):1327-36.*

1.3.4 Regulación de la hemostasia

Con el fin de limitar la coagulación al sitio y a una situación específica, se hace imprescindible que este proceso esté regulado. La regulación del mecanismo de coagulación ocurre a todos los niveles gracias a una serie de inhibidores que actúan en diferentes puntos.

En condiciones normales en la que no existe lesión alguna, el propio flujo sanguíneo aclara y reduce la concentración de sustancias con actividad coagulante previniendo el proceso de coagulación. Además, el propio endotelio vascular tiene una actividad antiagregante, anticoagulante y profibrinolítica gracias a distintas sustancias como el óxido nítrico, prostaciclina y el factor hiperpolarizante (EDHF) que inhiben la contracción, proliferación y agregación plaquetaria.

El TFPI es una serín proteasa con carga negativa que es producida por el endotelio sano, almacenada en un 10% en las plaquetas y que es liberada cuando éstas se activan. El TFPI regula al FT neutralizando la actividad catalítica del FXa, uniéndose a él y formando un complejo TFPI-FXa. Así se origina un complejo cuaternario constituido por TFPI-FXa-FT-FVIIa que es funcionalmente inactivo.

La antitrombina-III (AT-III) es una glicoproteína sintetizada en el hígado que inhibe a la trombina libre, pero su función inhibitoria es prácticamente nula sobre la trombina unida en la superficie plaquetaria. Por tanto, la AT-III actúa sobre la trombina, el FXa, FIXa, FXIa, FXIIa y sobre otras proteasas que estén alejadas de la zona de activación. La inhibición de la trombina por la AT está potenciada por los glicosaminoglucanos presentes, como el heparán sulfato y heparinas [404].

La proteína C1, además de pertenecer al sistema del complemento, es capaz de inhibir al FXIIa, al FXIa y a las calicreínas mediante la formación de complejos. La α1-antitripsina, sintetizada en el hígado, inhibe al FXIa, a la plasmina y a la trombina.

Por otro lado, la trombina, que escapa de la zona lesionada, se une a la trombomodulina anclada a la superficie endotelial formando el complejo trombina-trombomodulina, el cual es capaz de activar a la proteína C que, unida a su cofactor, la proteína S, forman un complejo que inhibe al FVIIIa y al FVa, impidiendo así la formación del complejo tenasa y protrombinasa.

La PS también puede actuar de manera libre facilitando la interacción entre el FXa y el TFPI, mostrando una actividad anticoagulante.

El cofactor II de la trombina (HCII), sintetizado en el hígado, inhibe a la trombina al asociarse a ella y forma un complejo. Su acción se puede ver potenciada por la presencia de heparina.

Por último, la tPA al ser capaz de activar la fibrinolisis, puede influir en el crecimiento del coágulo. Por otro lado, la trombina puede inhibir la fibrinolisis a través de la activación de TAFI, que interfiere en la activación de la plasmina y, en última instancia, inhibe la lisis del coágulo formado [405].

1.4 TÉCNICAS GLOBALES DE LA FUNCIÓN HEMOSTÁTICA

1.4.1 Trombinografía automática calibrada

La trombinografía automática calibrada (CAT, *Calibrated Automated Thrombogram)* es una técnica que permite llevar a cabo una evaluación global de la hemostasia. Es un método fluorogénico desarrollado por Hemker en 2003 [406], capaz de cuantificar la trombina generada en una muestra de plasma tras la activación de la coagulación.

A la muestra de plasma a estudiar se le adiciona un sustrato fluorogénico específico de la trombina que, cuando se escinde por la acción catalítica de ésta, libera un producto fluorescente. El dispositivo registra la fluorescencia a lo largo del desarrollo de la reacción, permitiendo conocer la cinética de la generación de trombina en una muestra.

Los tres reactivos más utilizados para desencadenar la generación de trombina son:

- MP-Reagent: reactivo con alto contenido en fosfolípidos (4 µM), que permite evaluar la generación de trombina dependiente de la concentración de FT de la muestra.

- PRP-Reagent: reactivo que contiene FT (1 pM) y que permite evaluar la generación de trombina dependiente de la concentración de fosfolípidos de la muestra.

- PPP-Low Reagent: reactivo que contiene una mezcla de FT y fosfolípidos (1 pM y 4 µM, respectivamente), que permite evaluar la generación de trombina dependiente de los factores de coagulación.

El test de generación de trombina se lleva a cabo en placas de 96 pocillos y las mediciones se suelen hacer por triplicado. Los valores de fluorescencia a lo largo del tiempo se registran mediante un fluorímetro, con una longitud de onda de excitación de 390 nm y de emisión de 460 nm, manteniendo una temperatura de 37^{o}C durante toda la reacción. El equipo cuenta con su propio software llamado *Thrombinoscope®* v3.0 (Thrombinoscope BV, Maastricht, Holanda) que registra automáticamente el valor de la fluorescencia y lo convierten en la actividad de la trombina. Durante la reacción, se puede observar la gráfica característica del experimento, el trombograma.

Esta técnica presenta una serie de ventajas y desventajas, que se detallan en la tabla 13:

Ventajas	Desventajas
- La medición no se ve afectada por la turbidez de la muestra.	- El plasma emite su propia fluorescencia. - La antigüedad del filtro y el tiempo de uso de la lámpara, así como el color de la placa utilizada pueden interfenir en la medición. - Durante la reacción, se consume el 30-40% del sustrato, lo que hace que disminuya la velocidad de conversión del sustrato por concentración de trombina, pudiendo afectar al cálculo de ésta.

Tabla 13. Ventajas y desventajas del CAT.

Aunque este método tiene ciertas desventajas, éstas pueden ser solventadas mediante la medición en paralelo de la generación de trombina en pocillos que contienen el plasma problema con una concentración conocida del complejo α2-macroglobulina/trombina. Dicho complejo tiene la capacidad de escindir al sustrato fluorogénico sin afectar a los sustratos de la trombina.

El trombograma es la representación gráfica de la concentración de trombina que se genera (nM) en función del tiempo (min). Los parámetros calculados de manera automática por el software T*hrombinoscope* son los siguientes (Fig.24):

- El tiempo de latencia (LT): tiempo desde el inicio del test hasta alcanzar una concentración de trombina de 10 nM, expresada en min.

- El tiempo al pico: tiempo que transcurre desde el comienzo del test hasta alcanzar la máxima concentración de trombina generada, expresada en min.

- La altura al pico (Pico): concentración máxima de trombina alcanzada, expresada en nM.

- El potencial endógeno de trombina (ETP): trombina total generada a lo largo del test, expresada en nMxmin.

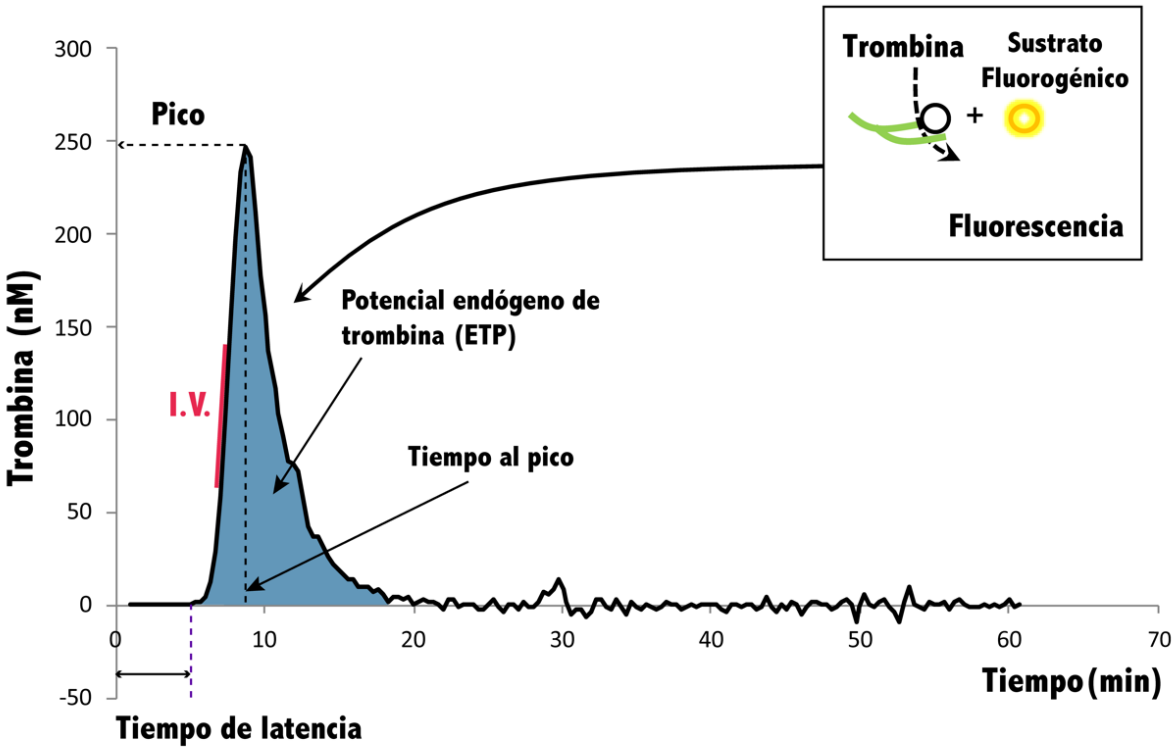


Figura 24. Representación de los parámetros del CAT.

A partir de los datos experimentales se puede calcular el índice de velocidad (IV), que permite evaluar la velocidad con la que se produce la trombina durante el test. Su cálculo se realiza aplicando la siguiente fórmula:

$$IV = \frac{AP}{TAP - LT}$$

El CAT ha demostrado ser útil en:

- La evaluación del riesgo trombótico
- La medición y la comparación del efecto de anticoagulantes
- El desarrollo de nuevos fármacos antitrombóticos
- La evaluación del riesgo de sangrado y el ajuste de dosis de los factores VIII ó IX en pacientes con hemofilia
- La detección de efectos secundarios de ciertos fármacos sobre la coagulación.

1.4.2 Tromboelastometría rotacional (ROTEM®)

La tromboelastometría rotacional (ROTEM®) es una técnica que evalúa la formación del coágulo, de manera global y continua, en una muestra de sangre completa anticoagulada con citrato de sodio, mediante el análisis de las propiedades viscoelásticas de la sangre durante la formación y la lisis del coágulo [407].

En este dispositivo, se emplea una cubeta que se fija en el canal de medición y donde se introduce la muestra de sangre completa junto con un activador de la coagulación. Dentro de la muestra, un pin rota sobre su propio eje. Con el tiempo, la sangre coagula y se produce una resistencia al movimiento del pin, en función de las propiedades viscoelásticas de la sangre. La amplitud de rotación del eje va disminuyendo a medida que el coágulo se hace más firme [408] (Fig. 25).

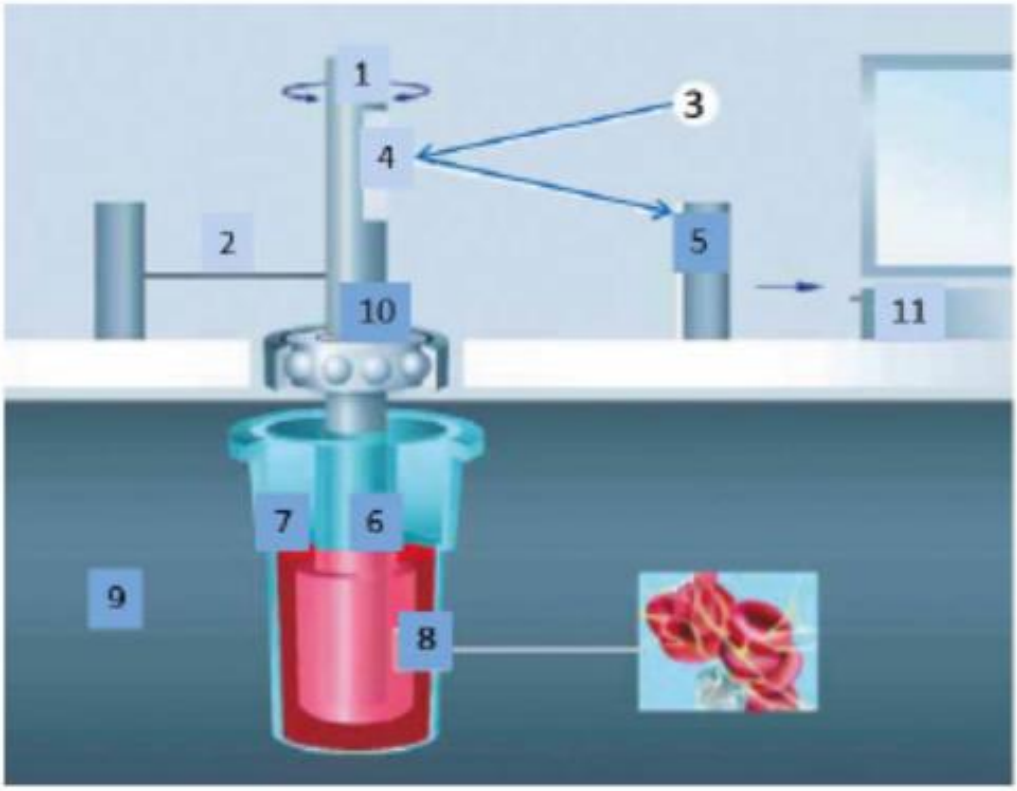


Figura 25. Componentes del ROTEM®. 1) Eje, 2) resorte, 3) fuente de luz, 4) espejo, 5) dispositivo de detección, 6) pin sensor, 7) cubeta para muestras, 8) fibras de fibrina y agregación plaquetaria, 9) portacubetas termostatizado, 10) rodamientos, 11) procesamiento de datos. Adaptado del Manual de Usuario de ROTEM® delta.

En la tabla 14 se presentan las ventajas y limitaciones de este sistema:

Ventajas	Limitaciones
- Realiza un estudio global de la hemostasia. - Informa sobre la interacción existente entre plaquetas – fibrinógeno independientemente del recuento plaquetario. - Sistema de pipeteo automático, lo que disminuye la probabilidad de error humano durante la prueba. - Puede predecir el riesgo de sangrado y de trombosis a largo plazo. - En pocos minutos se obtiene información para tomar decisiones terapéuticas rápidas.	- No analiza la contribución del endotelio en la coagulación, ya que se trata de un método *in vitro.* - Aunque se usa sangre completa, ésta está anticoagulada y recalcificada, por lo que no se puede analizar alteraciones de la hemostasia debido a acidosis y/o hipocalcemia. - No es capaz de determinar alteraciones en la coagulación debidas a hipotermias, ya que el test tiene lugar a 37ºC. - Las muestras se suelen obtener de vasos de la macrocirculación, por lo que no analiza la microcirculación, donde suelen comenzar las alteraciones hemostáticas.

Tabla 14. Ventajas y desventajas del ROTEM®.

La gráfica característica de esta prueba se conoce como tromboelastograma (Fig. 26), donde el eje X representa el tiempo, en min y el eje Y la amplitud del coágulo, en mm. Los parámetros que caracterizan la formación del coágulo son:

- Tiempo de coagulación (CT- *Clotting Time*, en s): tiempo que transcurre desde el comienzo del test hasta alcanzar una amplitud de 2 mm. Este parámetro depende de los niveles de los distintos factores de coagulación y de sus inhibidores y describe la rapidez de inicio de la formación de fibrina, que es también una medida de la velocidad de generación de trombina. La alteración de este parámetro podría indicar la presencia de niveles anómalos de factores de coagulación o de sus inhibidores y/o la presencia de alguna clase de contaminante, como heparina.

- Tiempo de formación del coágulo (CFT- *Clot Forming Time*, en s): tiempo desde la amplitud de 2 mm hasta una amplitud de 20 mm, en s. Describe la velocidad de formación del coágulo. Este parámetro depende, principalmente, de la cantidad de plaquetas y de su contribución en la firmeza del coágulo, así como del nivel de fibrinógeno y su polimerización.

- Ángulo-α: es el ángulo que forma la tangente de la curva de la coagulación, y junto al CFT, también describe la velocidad de formación del coágulo.

- Máxima fortaleza del coágulo (MCF-*Máximum Clot Firmness*, mm): mide la calidad del coágulo y representa la amplitud máxima que tiene el coágulo antes de comenzar la fibrinolisis. En este parámetro influyen las plaquetas, la concentración y capacidad de polimerización del fibrinógeno y los niveles de FXIII.

- Tiempo hasta la máxima fortaleza del coágulo (MCF-t, s): es el tiempo que transcurre desde el comienzo del test hasta alcanzar la máxima amplitud del coágulo.

- Amplitud a distintos tiempos (A10, 20…, mm): representan la firmeza del coágulo a distintos tiempos. En este parámetro influyen las plaquetas, la concentración y capacidad de polimerización de la fibrina y los niveles de FXIII.

- Lisis a distintos tiempos (LI30, 60…, %): expresa el porcentaje del coágulo que no se ha lisado a distintos tiempos desde el comienzo del test.

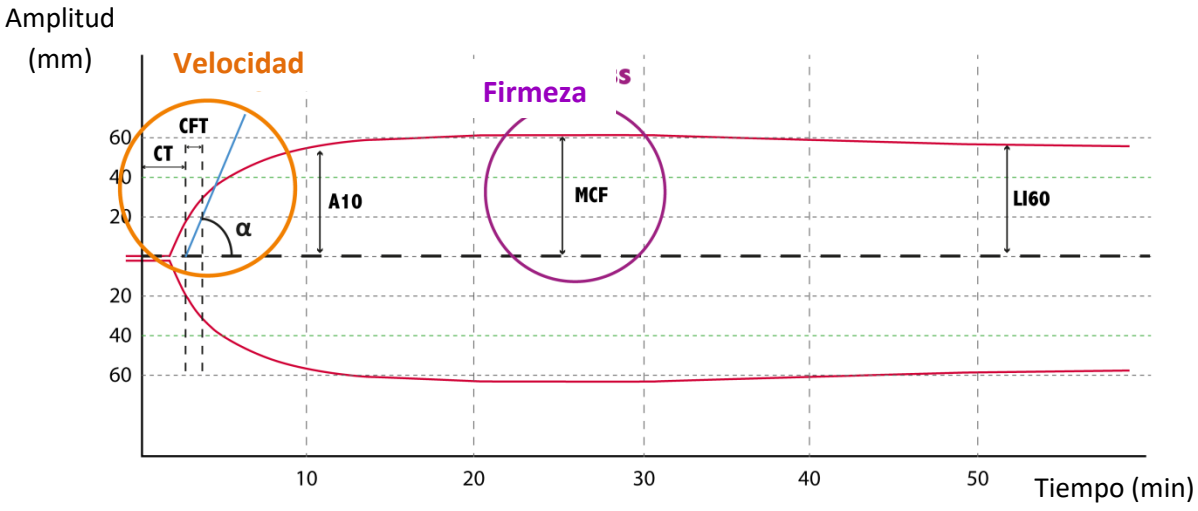
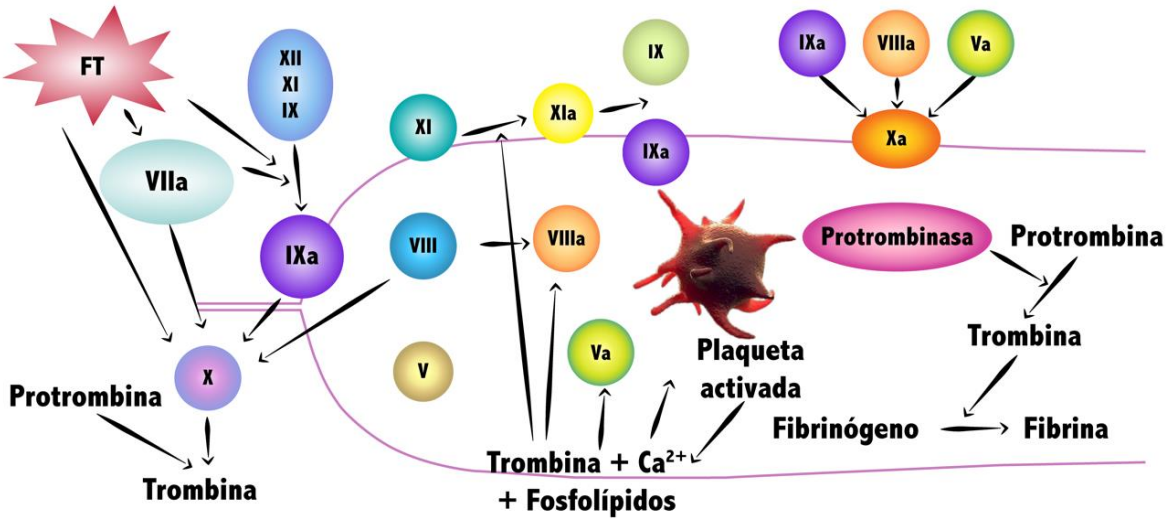


Figura 26. Representación de un tromboelastograma y sus distintos parámetros.

La figura 27 recoge los distintos factores que influyen en los diferentes parámetros del ROTEM® a lo largo de la formación del coágulo:



Parámetros del ROTEM®	CT- Tiempo de coagulación	Ángulo alfa CFT-Tiempo de formación del coágulo	AX-Amplitud a tiempo x MCF-Máxima fortaleza del coágulo	Ly(%)-%coágulo no lisado

Figura 27. Tromboelastograma y elementos participantes en sus distintas etapas. El inicio de la formación del coágulo está determinado por las plaquetas y los distintos factores de coagulación. La estabilidad del coágulo depende de las plaquetas y de la estabilización de la fibrina. Por último, la disolución del coágulo depende de la plasmina y otros mediadores fibrinolíticos. Adaptado de Kevin P. Blaine, Marc P. Steure. *Viscoelastic monitoring to guide the correction of perioperative coagulopathy and massive transfusion in patients with life-threatening hemorrhage*. Anesthesiology Clin., 2019, 37(1):51-66.

En la tabla 15 se recogen las distintas pruebas que se pueden realizar con el ROTEM® según los reactivos que se empleen y su aplicabilidad [409, 410]:

Test	Descripción e interpretación
NATEM	Recalcificación de la muestra y formación del coágulo sin necesidad de activación.
APTEM	Reactivo con aprotinina, para inhibir la fibrinólisis. Se compara con el test EXTEM para evaluar la fibrinólisis

Test	Descripción e interpretación
EXTEM	Reactivo con FT, fosfolípidos y un recalcificador. Permite evaluar la vía extrínseca de la coagulación. El test es sensible a los niveles de FVIII, FIX y FXI y permite también evaluar la vía común de la coagulación. El resultado se puede ver afectado por niveles de fibrinógeno anómalos y disfuncional, por defectos en la polimerización de la fibrina y en la función o número de las plaquetas. Permite detectar estados hiperfibrinolíticos.
INTEM	Reactivo con ácido elágico y fosfolípidos. El ácido elágico está cargado negativamente en las condiciones de pH a las que se realiza el test y permite evaluar la vía intrínseca de la coagulación. Test sensible al nivel de los factores de coagulación, a alteraciones de la función plaquetaria, a la polimerización de la fibrina y a defectos en la fibrinólisis.
FIBTEM	Reactivo con FT, $CaCl_2$ y citocalasina D, que inhibe la polimerización de la actina bloqueando la contribución de las plaquetas en la formación del coágulo. Los resultados dependen de los niveles de los factores de coagulación y de fibrinógeno. Al compararlo con el test EXTEM se puede estudiar la contribución del fibrinógeno en la fortaleza del coágulo.
HEPTEM	Reactivo con heparinasa que neutraliza la heparina no fraccionada de la muestra. Al compararlo con el test INTEM se puede evaluar el efecto de la heparina.

Tabla 15. Pruebas del ROTEM®.

Se han establecido intervalos de normalidad de cada parámetro del ROTEM® para cada tipo de test (Tabla 16).

Test	CT	CFT	Ángulo-α	A10	A15	A20	A25	MCF	Li30%	ML(%)
INTEM	100-240	30-110	70-83	44-66	48-69	50-71	50-72	50-72	94-100	<15
HEPTEM	La activación es como un INTEM, al que se añade heparinasa para inhibir la presencia de heparina o anticoagulantes derivados de la heparina en la muestra.									
EXTEM	38-79	34-159	63-83	43-65	48-69	50-71	50-72	50-72	94-100	<15
APTEM	La activación es como un EXTEM, al que se añade aprotinina para inhibir la fibrinolisis. Sirve para identificar estados de hiperfibrinolisis.									
FIBTEM	-	-	-	7-23	-	8-24	-	9-25	-	-
	- MCF < 9 mm es un signo de niveles bajos de fibrinógeno o polimerización del coágulo alterado. - MCF > 25 mm es un signo de niveles elevados de fibrinógeno (daría un EXTEM o INTEM normal, a pesar de estar ante un caso de trombocitopenia).									
NATEM	300-1000	150-700	30-70	-	-	35-60	-	40-65	94-100	<15

Tabla 16. Rangos de normalidad en las diferentes pruebas del ROTEM®.

2. HIPÓTESIS

2.HIPÓTESIS

Los pacientes con LES presentan manifestaciones clínicas muy variables, lo que sugiere que existen diferentes mecanismos fisiopatológicos implicados en el desarrollo de esta patología. Un alto porcentaje de los pacientes con LES presentan anticuerpos antifosfolípidos y estos anticuerpos serían los responsables de la activación basal de las plaquetas y del daño endotelial descrito en estos pacientes. Sin embargo, los pacientes con LES sin anticuerpos antifosfolípidos también podrían tener algún factor de riesgo cardiovascular no clásicos asociados a la enfermedad en el que podrían estar involucrados elementos plasmáticos y/o celulares.

El estudio del estado protrombótico de estos pacientes utilizando test globales de la coagulación (ROTEM® y CAT) podría aportar nuevos datos sobre aspectos fisiopatológicos de la enfermedad y ser útiles en el seguimiento de la evolución de pacientes con LES.

3. OBJETIVOS

3.OBJETIVOS

Los objetivos principales de este proyecto fueron:

- Caracterizar la hemostasia de los pacientes con LES que no poseen anticuerpos anti-FLs para independizarnos del efecto de los mismos
- Identificar los mecanismos involucrados en el desarrollo del perfil procoagulante observado en estos pacientes y su relación con la actividad de la enfermedad
- Evaluar la utilidad de la incorporación de los test globales de la coagulación en la práctica clínica habitual

4. MATERIALES Y MÉTODOS

4.MATERIALES Y MÉTODOS

4.1 DISEÑO DEL ESTUDIO

Se trata de un estudio transversal, prospectivo, de casos-controles. El estudio consiste en comparar las características de la hemostasia de un grupo de pacientes con LES sin anticuerpos anti-FLs con un grupo de controles sanos.

4.2 SUJETOS DEL ESTUDIO

62 pacientes diagnosticados de LES de la Unidad de Reumatología del Hospital General Universitario Gregorio Marañón (HGUGM) y de la Unidad de Medicina Interna del Hospital Universitario La Paz (HULP) fueron invitados a participar en el estudio entre los años 2013 y 2019. El grupo control estuvo formado por 88 sujetos sanos, reclutados en la Unidad de Donantes de Sangre del HULP. El protocolo del estudio fue aprobado por el Comité Ético del HGUGM y ratificado por el Comité Ético del HULP. Los sujetos que participaron en el estudio fueron incluidos tras la firma del consentimiento informado. Todos los procedimientos se realizaron de acuerdo con la declaración de Helsinki.

La información relativa a los participantes se trató respetando la legislación española sobre códigos éticos de conducta (Ley Orgánica de Protección de Datos Personales y garantía de los derechos digitales 3/2018, de 5 de diciembre, y la Ley 14/2007 de investigación biomédica y el Real Decreto 1716/2011).

El diagnóstico de LES se realizó siguiendo los criterios del ACR. La actividad de la enfermedad fue determinada por la escala SLEDAI-2K.

Son criterios de inclusión

- Criterios de inclusión para los pacientes con LES:
    - Cumplir al menos 4 criterios ACR para el diagnóstico de LES, revisión de 1997 [13]
    - Edad ≥ 18 años
    - Presencia de autoanticuerpos positivos (anti-ANA≥ 1:80 y/o anti-dsDNA ≥ 30 UI/ml)
    - Pacientes en régimen de tratamiento estable para LES en los últimos 30 días

- Criterios de inclusión para el grupo de controles sanos:

- Edad ≥ 18 años
- Controles que hayan aceptado participar en el estudio y hayan firmado el Consentimiento Informado

Son criterios de exclusión (para pacientes y controles sanos)

- Menopausia
- Abuso de alcohol
- Infección por VHC o VIH
- Tratamiento con fármacos con actividad antiplaquetaria y/o anticoagulante en la semana anterior al estudio
- Tratamiento con anticonceptivos orales o terapia hormonal (excepto tratamiento inmunosupresor con esteroides para el tratamiento de LES)
- Historia de infarto agudo de miocardio, angina o enfermedad coronaria, para evitar la inclusión de pacientes que quizás puedan presentar alguna condición clínica que sea factor de riesgo para ECV pero que no esté relacionada con LES
- Pacientes con anticuerpos anti-FLs (AL, anti-β2GP1 y anti-cL)
- Pacientes con diabetes, hipertensión arterial o hiperlipemia mal controladas
- Presencia de obesidad definida por un IMC > 30 kg/m^2
- Fumadores en los 12 meses previos al estudio
- Presencia de alguna enfermedad inflamatoria crónica, cáncer u otra patología que a juicio del investigador pueda afectar a los objetivos del estudio
- Parto o embarazo en los 3 meses previos al estudio

4.3 PREPARACIÓN DE LAS MUESTRAS

La hora de extracción de las muestras fue la misma para todos los sujetos del estudio (entre las 8 y 10 horas de la mañana) con el fin de evitar la influencia de los ciclos circadianos.

A cada sujeto se le extrajeron por venopunción antecubital, 21,6 mL de sangre total: 16,4 mL en tubos con citrato sódico 3,8%, 2,5 mL en tubos que contenían un inhibidor del FXIIa, el "*corn trypsin inhibitor"* (*CTI,* Cell Systems Biotechnologie, Troisdorf, Germany), y 2,7 mL en tubos de ácido etilendiaminotetraacético (EDTA, Vacutainer®, BD, Becton Dickinson and Company, Madrid, España).

Las muestras extraídas en tubos con EDTA se utilizaron para aislar a los neutrófilos y las extraídas en CTI para testar la dependencia de la generación de trombina asociada a las NETs con la fase de contacto de la coagulación. El resto de experimentos se realizaron con muestras extraídas en tubos de citrato.

El plasma rico en plaquetas (PRP) se obtuvo por centrifugación de sangre total (150 g, 10 minutos a 23ºC). Para obtener plasma libre en plaquetas (PFP), el PRP fue centrifugado dos veces más, la primera a 1500 g durante 15 minutos a 23ºC, y una última vez a 13000 g durante 2 minutos, a 23ºC.

Las alícuotas de PPP y de PFP fueron almacenadas a -80ºC hasta el momento de su análisis.

4.4 ANÁLISIS DE LAS MUESTRAS

Recuento plaquetario y celular.

El recuento plaquetario y celular se llevó a cabo con el contador automático de Coulter Ac. T Diff cell counter (Beckman Coulter, Madrid, España). Los niveles de las proteínas del complemento C3 y C4 y de las inmunoglobulinas de los pacientes, así como la creatinina, la proteinuria a las 24h, la VSG y el título de anticuerpos se determinaron solo en pacientes, en el laboratorio central del HGUGM y del HULP.

Determinación de los niveles plasmáticos de E-selectina soluble, PAI-1 y LDL-oxidada

Los niveles de E-Selectina soluble (sE-Selectina), (R&D systems Europe Ltd., Abingdon, UK), PAI-1 (Invitrogen, Viena, Austria) y LDL-oxidada (Mercodia, Uppsala, Sweden) fueron determinados en PFP mediante el uso de kits ELISA comerciales y medidos en un fotómetro para microplacas Multiskan FC (ThermoScientific, Madrid, Spain).

Evaluación de la función plaquetaria

El estudio de la activación plaquetaria se realizó en estado basal y después de su estimulación con dos agonistas diferentes. El PRP de las muestras obtenidas se diluyó con tampón HEPES (10 mM HEPES, 145 mM NaCl, 5 mM KCl, and 1 mM $MgSO_4$, pH 7,4) a una concentración 1:4.

Las alícuotas de 50 µL de PRP diluido se incubaron con el péptido activador del receptor de trombina 6 a 100 µM (TRAP, Bachem, Suiza) o con difosfato de adenosina a 20 µM (ADP, Sigma, Madrid) durante 10 minutos a temperatura ambiente (TA).
Tras la incubación con el activador, se añadió el anticuerpo monoclonal (mAb) PAC1 (Becton Dickinson, Madrid, España) que reconoce únicamente la forma activa del receptor de fibrinógeno o el mAb contra la P-selectina humana, que actúa como marcador de gránulos-α (BD Pharmingen, San Diego, CA, USA). Ambos mABs estaban marcados con fluoresceína (FITC) y se dejaron incubar en la oscuridad durante 15 minutos a TA.

Como control de los gránulos densos se utilizó un mAB contra la granulofisina (CD63, gp55) tras la activación del PRP con TRAP (BD Pharmingen, San Diego, CA, USA). Las muestras se dejaron incubar con este mAB marcado con FITC, 15 minutos a TA en la oscuridad.

Con el fin de determinar el número de receptores de fibrinógeno existentes en la superficie de las plaquetas, el PRP se incubó 20 minutos en oscuridad a TA, con un mAb dirigido específicamente contra la subunidad αIIb (Biocytex, Marsella, Francia) marcado con ficoeritrina (PE) o con un mAb dirigido contra la subunidad β3 del receptor de fibrinógeno marcado con FITC (BD, Pharmingen, Madrid, España).

Para determinar el número de receptores del FvW existente en la membrana plaquetaria, el PRP también fue incubado, sin agonistas, con mAbs dirigidos contra la subunidad GPIX y GPIbα de dicho receptor (BD, Pharmingen, Madrid, España).

Finalmente, las plaquetas de todas las muestras fueron diluidas de nuevo con tampón HEPES (1:6) y se analizaron por citometría de flujo (CMF) utilizando el citómetro de flujo FACscan (BD biosciences). El software utilizado fue el CellQuest ProTM (BS Biosciences).

Exposición de PS en la superficie de plaquetas

El estudio de la apoptosis plaquetaria se llevó a cabo mediante la evaluación de la exposición de PS en la superficie de plaquetas lavadas, mediante la unión de anexina V marcada con FITC (Becton Dickinson, Madrid, España).

Para obtener plaquetas lavadas, se añadió al PRP ácido cítrico-dextrosa (citrato de sodio 85 mM, ácido cítrico 65 mM y glucosa 104 mM). Posteriormente se realizó una centrifugación a 650 g,

durante 10 minutos a 23ºC. El pellet se resuspendió en un volumen similar de tampón HEPES (10 mM HEPES, 145 mM NaCl, 5 mM KCl y 1 mM $MgSO_4$; pH 7,4).

Finalmente, a las plaquetas lavadas se les añadió el tampón de unión del kit de anexina V (10 mM HEPES, 10 mM NaOH, 140 mM NaCl, 2,5 mM $CaCl_2$, pH 7,4) y anexina V-FITC. Tras incubar las muestras durante 15 minutos en oscuridad y a TA, las muestras fueron analizadas por CMF.

Evaluación de la actividad de las caspasas-3/7, -8 y -9

Para evaluar la actividad de las caspasas se utilizó PRP diluido 1:10 con tampón HEPES enriquecido con calcio (150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM HEPES, pH 7,42) con 2 mM Gly-Pro-Arg-Pro (Sigma, Madrid), para evitar la formación de agregados.

El análisis de la actividad de las caspasas se llevó a cabo utilizando inhibidores de caspasas conjugados con un fluorocromo (fluorochrome-labeled inhibitors of caspases, FLICA). Cada FLICA contiene tres dominios: un dominio fluorocromo (carboxifluoresceína, FAM), un dominio de reconocimiento de la caspasa que contiene 3 ó 4 aminoácidos (DEVD, para caspasas- 3/7, LETD para caspasa-8 y LEHD para caspasa-9) y, por último, un dominio de unión covalente consistente en cloro-/fluoro-metil cetona (FMK), el cual se une covalentemente y de manera irreversible a la cisteína de la caspasa correspondiente.

Los FLICAs, que no son tóxicos, penetran en la célula a través de la membrana plasmática y se unen a las caspasas correspondientes. Las muestras de PRP se dejaron incubar 1 hora con los FLICAs para la caspasas-3/7, -8 y -9 (Millipore, Madrid, España). Finalmente, se diluyeron 1:6 con PBS y se determinó la actividad de las caspasas por CMF.

Evaluación de los agregados linfocitos-plaquetas

Con el fin de determinar la formación de agregados entre las plaquetas y los linfocitos, la sangre completa se diluyó 1:10 con tampón HEPES (KCl 5mM, HEPES 10 mM, $MgSO_4$ 1mM, NaCl 150 mM, ajustado a pH 7,4) y se incubó con los anticuerpos CD41-PE (Biocyte, Marsella, Francia), específico de plaquetas, y CD45-FITC (Becton Dickinson, Madrid, España), específico de linfocitos.

Además de evaluar la formación de agregados en condiciones basales, también se estudió tras la estimulación con TRAP (50 µM) o ADP (40 µM), durante 15 minutos en oscuridad y a TA. Finalmente, las muestras se diluyeron 1:6 con PBS y se analizaron mediante CMF. Los agregados

formados entre plaquetas y linfocitos se definieron como la fluorescencia de linfocitos marcados con $CD41^{+}$-PE.

Formación de NETs

Para generar NETs, se aislaron los neutrófilos de los pacientes y de los controles a partir de muestras de sangre completa, que fueron diluidas primero 1:1 con PBS y por segunda vez al 50% con Percoll al 65%. Las muestras se centrifugaron a 500 g durante 25 min a 5ºC. Se realizaron varios lavados con PBS y agua con el fin de romper y eliminar los eritrocitos.

Una vez aislados los neutrófilos y tras su contaje con la cámara de Neubauer, $2{,}5x10^{5}$ neutrófilos se cultivaron con medio RPMI (Invitrogen, Madrid, España) y, si procedía, se estimularon con PMA 100 nM (Sigma, Madrid) durante 30 min a 37ºC para someterlos al proceso de NETosis (Fig. 28).

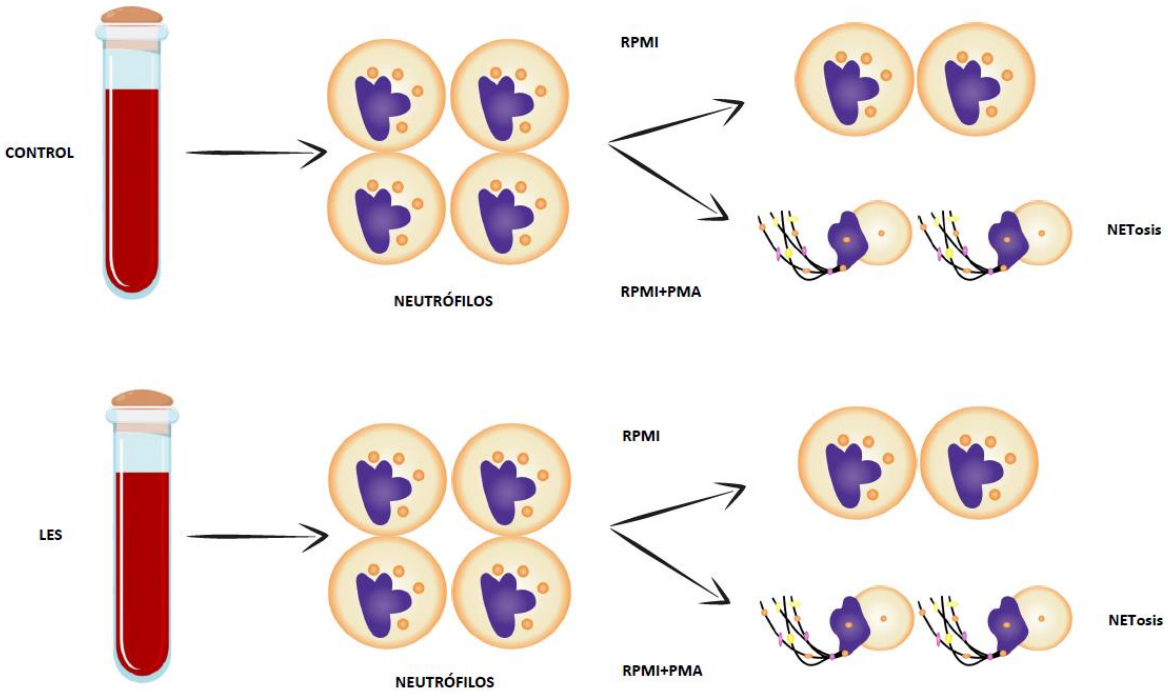


Figura 28. Esquema del tratamiento de los neutrófilos una vez aislados.

Preparación de muestras de NETs para microscopía

Se sembraron $5x10^{5}$ neutrófilos en 500 µL de medio RPMI sobre cubreobjetos de cristal de 12-mm pretratados con poly-L-lisina (Sigma-Aldrich, Suecia) en placas de 25 pocillos. Si procedía, las muestras eran tratadas con PMA 100 nM, durante 30 min a 37ºC. Posteriormente se fijaron con paraformaldehído al 2% de concentración final, durante 15 min a TA. Los portas se bloquearon

añadiendo PBS-BSA 2% + suero de cabra 2% durante 15 min a TA. Después, las muestras fueron incubadas con un anticuerpo primario contra la Mieloperoxidasa humana (*DAKO, Madrid, España*) diluido 1:300 con PBS-BSA 2% durante 1 hora. El anticuerpo secundario, conjugado con el fluorocromo Alexa fluor 488 (ThermoFisher Scientific, Madrid, España) y diluido 1:500 con 2% BSA-PBS, se agregó durante 45 min a TA.

Por último, las muestras fueron embebidas en medio de montaje con DAPI (Laboratorios Vector, Burlingame, CA, USA) y mantenidas a 4ºC en oscuridad hasta su posterior observación por microscopía de fluorescencia, usando un microscopio Nikon Eclipse 90i.

Cuantificación del ADN-libre

El cfDNA fue determinado en PFP mediante el kit Quanti-IT™ PicoGreen dsDNA assay (Thermofisher Scientific, Waltham, MA, USA), siguiendo las instrucciones del fabricante. Posteriormente, se midió la fluorescencia en un lector multi-modal de microplacas Synergy 4 (Biotek, EEUU), a una longitud de onda de excitación de 480 nm y 520 nm de emisión.

4.5 EVALUACIÓN DE LA HEMOSTASIA MEDIANTE TÉCNICAS GLOBALES DE LA COAGULACIÓN

Trombinografía automática calibrada

La generación de trombina se evaluó mediante trombinografía automática calibrada (CAT).

El CAT se llevó a cabo con muestras de PFP descongeladas a 37ºC durante 10 min. Determinamos la generación de trombina asociada al plasma con el reactivo PPP-LOW Reagent (1 pM FT y 4 µM fosfolípidos), al FT de las MPs con el reactivo MP-Reagent (4 µM fosfolípidos), y a los fosfolípidos de las MPs con PRP-Reagent (1 pM FT). Para esto, se añadieron 40 µL de las muestras de PPP a cada pocillo junto con 10 µL del reactivo y 10 µL de solución tampón que contiene el sustrato específico de la trombina y $CaCl_2$ (solución FluCa Kit). Todos los reactivos son de Diagnostica Stago, Madrid.

Los valores de fluorescencia a lo largo del tiempo se registraron mediante un fluorímetro (FLuorScan), con una longitud de onda de excitación de 390 nm y de emisión de 460 nm. Estos valores se convirtieron en actividad de la trombina con el tiempo mediante un programa específico llamado Thrombinoscope (*Thrombinoscope BV, Maastricht, Holanda*) versión 3.6. Todas las muestras se midieron por triplicado.

Mediante el CAT se registraron los parámetros referentes al LT, tiempo al pico, pico y ETP. Posteriormente se calculó el IV.

Tromboelastometría rotacional (ROTEM®)

El ROTEM® se realizó en sangre completa de muestras que se mantuvieron en reposo durante 30 minutos antes de la medición y atemperados a 37ºC. Para estudiar la cinética de formación del coágulo se realizó el test NATEM® (Non-activated test, $CaCl_2$) que evalúa la activación endógena de la hemostasia.

De forma paralela, se llevó a cabo el test FIBTEM (citocalasina D, FT y $CaCl_2$), que elimina la contribución plaquetaria en la formación del coágulo.

La presencia de $CaCl_2$ (0,2 M) en ambos reactivos permite revertir el efecto anticoagulante del citrato de sodio contenido en los tubos de extracción sanguínea. Los test se llevaron a cabo añadiendo 300 µL de sangre completa y 20 µL de los reactivos ya mencionados anteriormente, dependiendo del tipo de test que se llevase a cabo.

Los parámetros determinados en el test NATEM fueron el CT, CFT, ángulo-α, MCF, MCF-t, A5, A10 y A15 y se realizó el estudio de la fibrinolisis con los parámetros Li30, Li60 y Li90, mientras que en el test FIBTEM se analizó el parámetro MCF.

Evaluación de la generación de trombina asociada a las NETs

La generación de trombina se evaluó en el CAT en presencia de neutrófilos estimulados con PMA (NETs) y no estimulados. Para ello, a cada pocillo de la placa se le añadió 40 µL de neutrófilos previamente cultivados con PRP ajustado a 10^5 plaquetas/µL de controles, con y sin CTI y 10 µL de FluCa (Fig. 29). No fue necesario la adición de ningún reactivo para desencadenar la reacción. Las determinaciones se realizaron por duplicado.

En el CAT se determinaron el LT, pico, tiempo al pico y ETP. Posteriormente se calculó el IV.

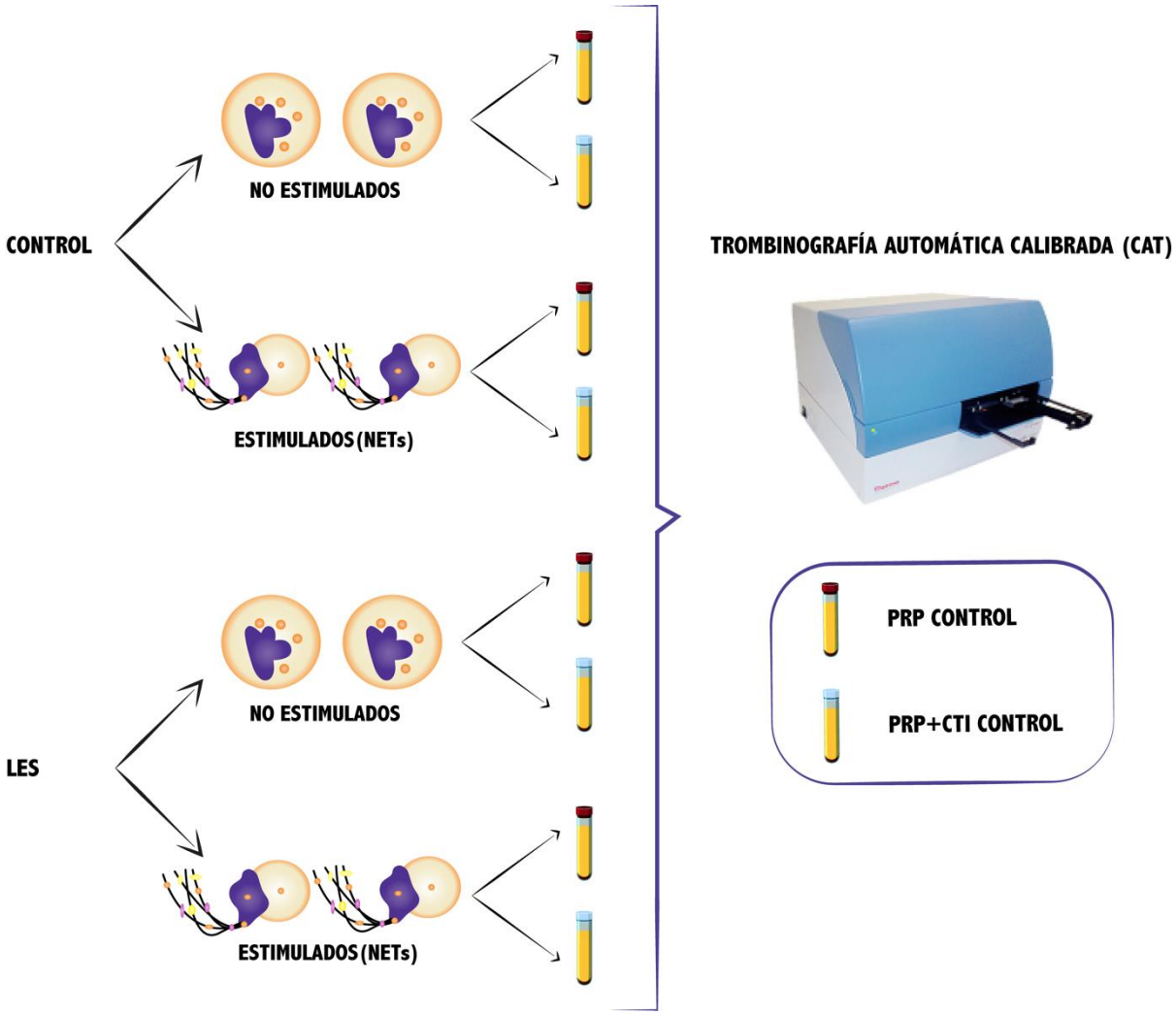


Figura 29. Esquema representativo de la evaluación de la generación de trombina asociada a las NETs.

4.6 ANÁLISIS ESTADÍSTICO

Se calcularon las medidas de tendencia central y dispersión de los datos en el caso de variables cuantitativas, y la frecuencia absoluta y relativa en el caso de variables cualitativas.

La comparación entre dos grupos independientes se llevó a cabo mediante la t-Student, en caso de tener una distribución normal o test de Mann-Whitney en caso de datos que no tengan una distribución normal.

Para estudiar la correlación entre variables se utilizó Pearson en el caso de datos paramétricos y Spearman en el caso de datos no paramétricos. Los datos fueron expresados como la media ± desviación estándar en el caso de datos con distribución normal o como la mediana (percentil 25-percentil 75) en datos que no siguiesen una distribución normal.

Los análisis estadísticos se llevaron a cabo utilizando el paquete estadístico GraphPad Prism v.5.03 (GraphPad Software Rockfield, CA, USA). Todos los análisis estadísticos fueron considerados bilaterales y los valores de $p<0,05$ se consideraron estadísticamente significativos.

5. RESULTADOS

5.1 CARACTERÍSTICAS CLÍNICAS Y DEMOGRÁFICAS DE LA POBLACIÓN A ESTUDIO

El estudio se llevó a cabo en 32 pacientes diagnosticados de LES según los criterios de ACR tratados en la Unidad de Reumatología del HGUGM y en la Unidad de Medicina Interna del HULP. Además, participaron 88 controles sanos, que fueron reclutados en el Centro de Donación de Sangre del Hospital Universitario La Paz. El estudio se realizó en el periodo comprendido entre enero 2013 y diciembre 2019. Ningún paciente presentó signos o síntomas de trombosis en el momento de la inclusión.

La tabla 17 recoge los pacientes con LES incluidos en el estudio, su edad, el tiempo transcurrido desde el diagnóstico de la enfermedad, el tratamiento en el momento de la inclusión, así como las enfermedades concomitantes y el índice SLEDAI-2K de cada uno.

Paciente	Evolución de la enfermedad (años)	Edad (Años)	Medicación en el momento del estudio	Enfermedades concomitantes	SLEDAI-2K
1	23	44	Sin tratamiento		12
2	21	50	Omeprazol, micofenolato de mofetilo, prednisona, calcifediol	Trombocitopenia autoinmune	6
3	9	35	Tramadol, levotiroxina, prednisona, rituximab	Síndrome de Sjögren, enfermedad de Graves-Basedow, hepatitis autoinmune, fibromialgia	3
4	18	35	Ramipril, fenelzina, abatacept, inmunoglobulinas		4
5	26	32	Sin tratamiento	Síndrome de Raynaud, endometriosis	1
6	19	31	Sin tratamiento	Fotosensibilidad, artralgias inflamatorias, linfopenia	5

Paciente	Evolución de la enfermedad (años)	Edad (Años)	Medicación en el momento del estudio	Enfermedades concomitantes	SLEDAI-2K
7	13	49	Sulfato ferroso, omeprazol, prednisona, belimumab, azatioprina	Síndrome de Sjögren	4
8	19	35	Quetiapina, duloxetina, omeprazol, diazepam, hidroxicloroquina, azatioprina, pregabalina, prednisona, tramadol, sulfato ferroso, calcifediol, rituximab		2
9	27	57	Prednisona, azatioprina, belimumab, rituximab		14
10	12	46	Calcifediol		2
11	4	45	Propionato de clobetasol, trazodona, calcipotriol, diazepam, hidroxicloroquina, calcifediol, pregabalina, metamizol, omeprazol, almotriptán, enalapril, prednisona, sertralina, micofenolato de mofetilo, abatacept, belimumab	Síndrome de Raynaud, nefropatía lúpica, dislipidemia mixta	2

Paciente	Evolución de la enfermedad (años)	Edad (Años)	Medicación en el momento del estudio	Enfermedades concomitantes	SLEDAI-2K
12	10	29	Azatioprina, hidroxicloroquina, prednisona, omeprazol, calcifediol, sulfato ferroso, belimumab	Síndrome de Sjögren, leucopenia/linfopenia	7
13	2	45	Hidroxicloroquina, prednisona, calcifediol, levotiroxina, azatioprina		0
14	15	33	Hidroxicloroquina, azatioprina, condroitín sulfato		4
15	3	21	Hidroxicloroquina		4
16	23	61	Symbicort, enalapril, tramadol, pregabalina, azatioprina		0
17	10	31	Sin tratamiento		4
18	11	35	Metotrexato, omeprazol, ácido fólico, prednisona, micofenolato de mofetilo		2
19	22	41	Nifedipina, hidroxicloroquina	Enfermedad mixta del tejido conectivo, síndrome de Raynaud	2
20	24	56	Abatacept, furosemida, fluoxetina, lorazepam, amisulprida, omeprazol, prednisona, espironolactona	Artritis reumatoide, síndrome de Sjögren, hepatitis autoinmune	4

Paciente	Evolución de la enfermedad (años)	Edad (Años)	Medicación en el momento del estudio	Enfermedades concomitantes	SLEDAI-2K
21	6	67	Hidroxicloroquina, calcifediol		0
22	8	62	Hidroxicloroquina, omeprazol, levotiroxina, diazepam, paroxetina		2
23	23	58	Hidroxicloroquina, prednisona, atenolol, calcifediol		4
24	7	25	Hidroxicloroquina, calcifediol, robaxisal		10
25	4	65	Hidroxicloroquina, prednisona, atorvastatina	Hipertensión arterial	8
26	9	40	Micofenolato de mofetilo, prednisona, hidroxicloroquina, enalapril, denosumab, ranitidina, paroxetina, calcifediol		0
27	8	22	Micofenolato de mofetilo, ácido ursodesoxicólico, hidroxicloroquina, calcifediol, ranitidina	Alfa-talasemia minor, síndrome de Raynaud, hiperhidrosis secundaria	4
28	22	48	Hidroxicloroquina, prednisona, calcifediol, omeprazol, colecalciferol		2
29	12	40	Calcifediol		2
30	18	33	Prednisona, hidroxicloroquina, calcifediol	Aneurisma del septo atrial, enfermedad de Kikuchi-Fujimoto, osteonecrosis	2

Paciente	Evolución de la enfermedad (años)	Edad (Años)	Medicación en el momento del estudio	Enfermedades concomitantes	SLEDAI-2K
31	37	52	Omeprazol, hidroxicloroquina, amitriptilina, calcifediol	Depresión	4
32	15	38	Hidroxicloroquina		0

Tabla 17. Características clínicas de los pacientes con LES.

Respecto a la edad, no se observaron diferencias significativas entre ambos grupos (tabla 18).

	Controles	LES	p-valor
Edad (años)	38,3 ± 11,6	41,9 ± 12,9	0,0893

Tabla 18. Media de edad de los distintos grupos. Datos expresados como media ± desviación estándar.

5.2 PARÁMETROS BIOQUÍMICOS: RECUENTO CELULAR Y MARCADORES RELACIONADOS CON LA HEMOSTASIA Y ACTIVACIÓN ENDOTELIAL

En los pacientes con LES se observaron niveles disminuidos de linfocitos, eritrocitos, granulocitos, leucocitos, plaquetas y hematocrito, en comparación al grupo control. A pesar de esta diferencia, los recuentos celulares se encontraban dentro del rango de normalidad (tabla 19).

Parámetro	Controles	LES	p-valor	Rango de normalidad
Linfocitos/µL	1,9 (1,6–2,4)	1,6 (1–1,8)	0,0093*	1,2–3,4
Eritrocitos x10^6/µL	4,3 (4,1–4,6)	4,1 (3,8–4,4)	0,0421*	4–6
Monocitos x10^3/µL	0,4 (0,3–0,5)	0,4 (0,2–0,4)	0,1046	0,1–0,6
Granulocitos x10^3/µL	4 (2,9–5,3)	2,9 (2,3–3,6)	0,0065*	1,4–6,5
Leucocitos x10^3/µL	6,4 (5,3–7,6)	4,8 (4,2–5,7)	0,0012*	4,5–10,5

Parámetro	Controles	LES	p-valor	Rango de normalidad
Hemoglobina (g/dL)	13,2 (12,3–14,2)	12,9 (11,6–13,5)	0,1226	11–18
Plaquetas x10^3/µL	247 (208–284)	194 (171,5–231)	<0,0001*	150–450
Hematocrito (%)	40,1 (38,4–43,7)	35,9 (34,4–39,8)	0,0004*	35–60
VCM (fL)	94,6 (91,5–96,9)	92,4 (88,1–97)	0,2407	80–99,9
HCM (pg)	30,3 ± 1,6	28,6 ±3,3	0,3037	27–31
CHCM (g/dL)	31,1 (31,3–32,9)	31,6 (30,7–32,3)	0,0976	33–37
ADE (%)	13,5 (12,8 –14,3)	14,1 (13,2–15,5)	0,1561	11,6–13,7
VPM (fL)	6,9 ± 0,8	7 ± 0,8	0,8931	7,8–11
Ptc (%)	0,17 (0,15–0,19)	0,14 (0,12–0,19)	0,1210	0,190–0,36
ADP (%)	17,1 ± 0,8	17,3 ± 0,9	0,4936	0,190–0,36
PCR (mg/dL)	ND	0,2650 (0,12–0,6)	-	0–0,5
C3 (mg/dL)	ND	88,4 (71,5–106)	-	75–135
C4 (mg/dL)	ND	16,3 (11,8–20,7)	-	14–60
Anti-DNA (mg/dL)	ND	14 (2,9–23)	-	<15,00
VSG (mm)	ND	10,77 ± 0,6	-	2–20
Creatinina(mg/dL)	ND	0,72 ± 0,12	-	0,5–0,9
IgG (mg/dL)	ND	1130 (910,5–1252)	-	725–1900
IgA (mg/dL)	ND	224,6 ±92,32	-	50–350
IgM (mg/dL)	ND	84 (61,6–107,5)	-	45–280

Tabla 19. Parámetros bioquímicos y recuento celular. Los datos fueron analizados mediante el test de Mann-Whitney o la t-Student y los resultados están expresados como la mediana (P25-P75) o media ± desviación estándar en función de la distribución de los datos, considerándose $p<0.05$ significativo e indicando diferencia significativa con *****. Abreviaturas: VCM, volumen corpuscular medio; HCM, hemoglobina corpuscular media; CHCM, concentración de hemoglobina corpuscular media; ADE, amplitud de distribución eritrocitaria; VPM, volumen plaquetario medio; Ptc, plaquetocrito; ADP, amplitud de distribución plaquetaria; PCR, proteína C reactiva; VSG, velocidad de sedimentación de eritrocitos; ND, no determinado.

5.3 NIVELES PLASMÁTICOS DE LDL-OXIDADA Y E-SELECTINA

Como en la literatura ha sido ampliamente descrita la relación existente entre la coagulación y el estado inflamatorio, se midieron los niveles séricos de E-selectina y LDL-oxidada. Los pacientes

con LES presentaron un incremento en los niveles de LDL-oxidada y E-selectina respecto a los controles (Fig.30).

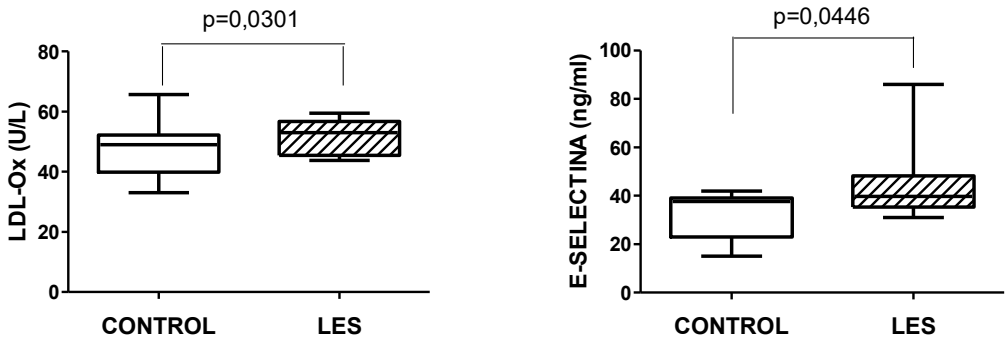


Figura 30. Niveles plasmáticos de LDL-ox y E-selectina. Los datos se analizaron mediante el test de Mann-Whitney. p<0,05 fue considerado significativo.

5.4 EVALUACIÓN DE LA CINÉTICA DE LA FORMACIÓN DEL COÁGULO *IN VITRO* MEDIANTE ROTEM®

Para evaluar la cinética de formación del coágulo y llevar a cabo el estudio de la hemostasia global, se realizó el ROTEM® a partir de sangre entera. Los resultados mostraron un perfil procoagulante en pacientes con LES en comparación al grupo control, ya que en el grupo de pacientes se encontraron valores significativamente mayores en los parámetros relacionados con la velocidad de formación del coágulo (evaluada por los parámetros NATEM-CT y NATEM-α), con un mayor ángulo-α y un acortamiento del CT en dicho grupo. Además, la MCF y el A15 también fueron significativamente mayores en los pacientes (Fig.31). Sin embargo, no se encontraron diferencias significativas en la lisis del coágulo a los 60 minutos (datos no mostrados).

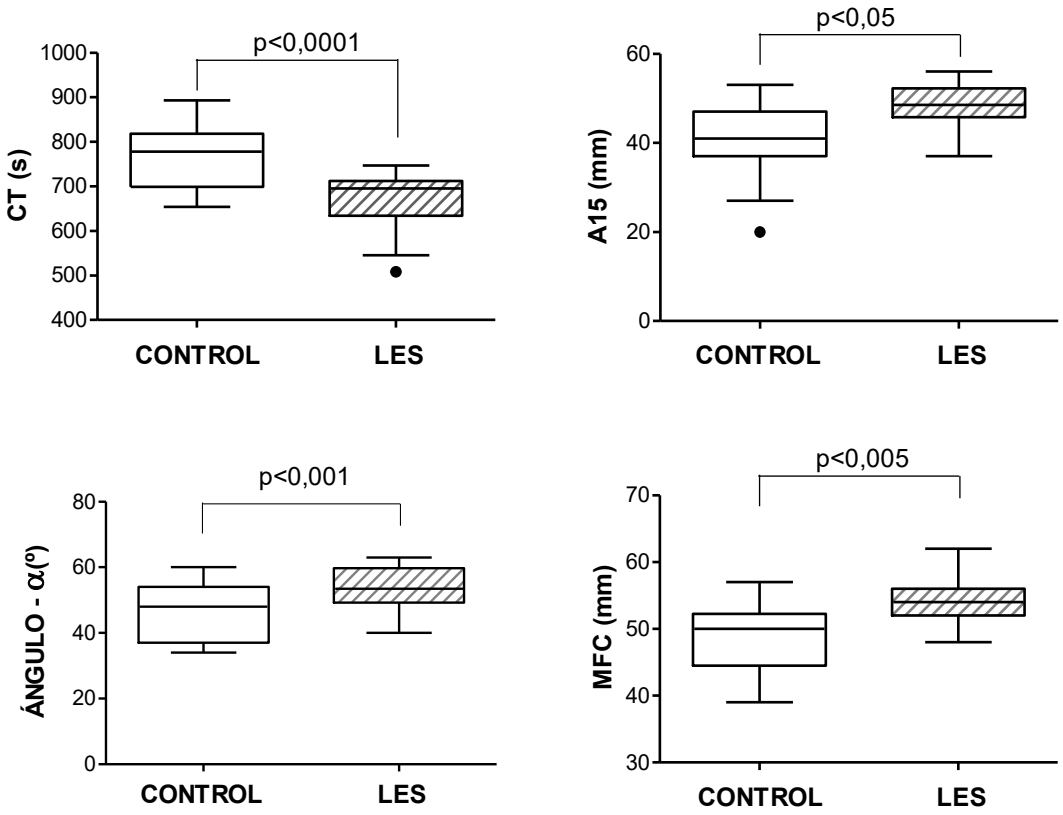


Figura 31. Resultados obtenidos en ROTEM®. La tromboelastometría rotacional se realizó con sangre completa, tal y como está detallado en el apartado de "Materiales y Métodos". Según la distribución de los datos, estos fueron analizados con el test de Mann-Whitney o con la t-Student, considerándose p<0.05 significativo. LES: lupus eritematoso sistémico. CT: tiempo de coagulación, A15: amplitud a los 15 min, MFC: máxima fortaleza del coágulo.

Por otro lado, los pacientes con LES también presentaron un incremento del MCF en el test FIBTEM en comparación a los controles (Fig. 32).

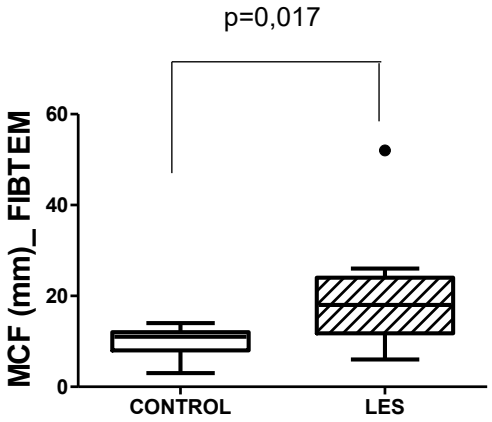


Figura 32. MCF en FIBTEM. Los datos fueron analizados con el test de Mann-Whitney y se estableció significancia con p<0,05. MCF: máxima fortaleza del coágulo.

Además, se realizó la prueba estadística de Kruskal-Wallis para determinar si la presencia de otras manifestaciones clínicas, como el síndrome de Raynaud y de Sjögren, podían tener un efecto sinérgico con la presencia intrínseca del LES en nuestra cohorte de estudio, en referencia a la formación del coágulo evaluada por ROTEM®. No se hallaron diferencias entre los pacientes con LES sin ningún otro síndrome, pacientes con LES y con síndrome de Sjögren y pacientes con LES y con síndrome de Raynaud (tabla 20).

	SOLO LES	LES CON SÍNDROME DE SJÖGREN	LES CON SÍNDROME DE RAYNAUD
Número de datos	20	4	7
CT	705,5 (666-712)	648,5 (537,5-728)	712 (678-728)
Ángulo-α	52,5 (49-58,5)	55,5 (50,5-61,25)	54 (43-60)
MCF	54 (49-55,75)	54,5 (52,25-56,75)	54 (54-57)

Tabla 20. Perfil procoagulante en pacientes LES, LES sin síndrome de Sjögren y LES sin síndrome de Raynaud. Los datos se representan con la mediana (percentil 25-percentil 75). El test Kruskal-Wallis junto con el test post-hoc Dunn no mostraron diferencias significativas entre los tres grupos.

Por otra parte, aún excluyendo los pacientes con LES y otras enfermedades autoinmunes, se siguió observando un perfil procoagulante en este subgrupo de pacientes con LES al compararlo con el grupo control (Fig. 33).

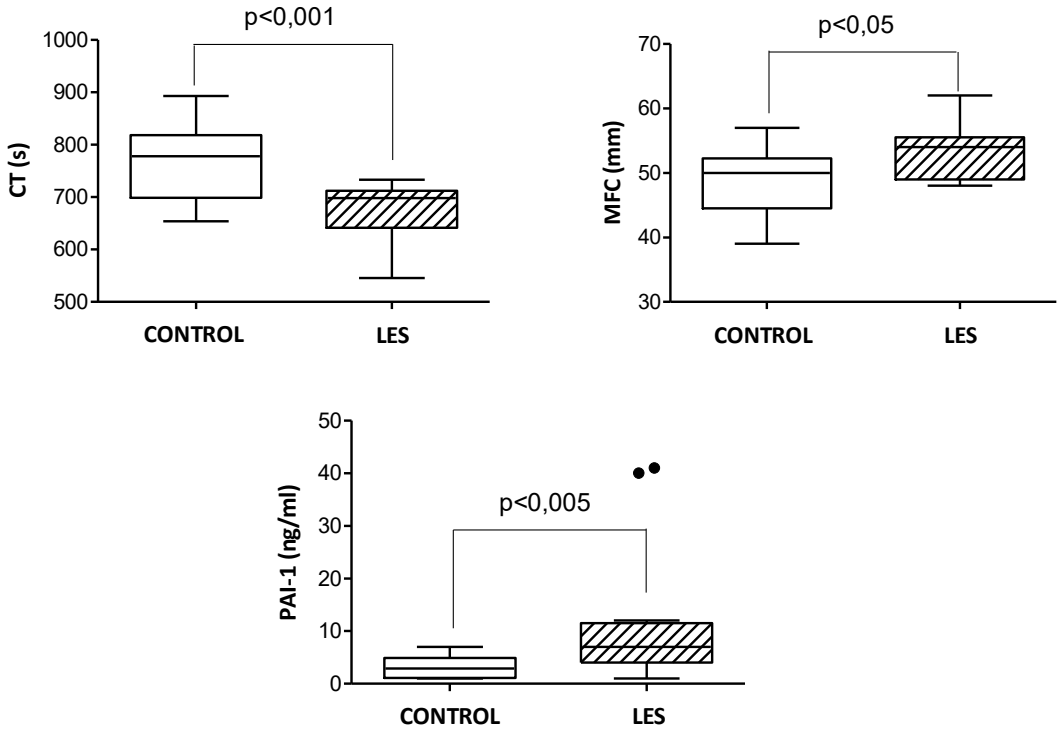


Figura 33. Perfil procoagulante en pacientes con LES sin otras enfermedades autoinmunes. Parámetros del ROTEM (CT y MFC) fueron determinados en los controles sanos y pacientes con LES sin otras enfermedades autoinmunes. Se realizó el test Mann-Whitney y se estableció significancia con p<0.05.

Si bien la media de los pacientes con LES presentaban un perfil procoagulante, en algunos de ellos los parámetros del ROTEM® daban valores que estaban dentro del rango de normalidad de los del grupo control. Con el objetivo de determinar si aquellos que tenían un marcado perfil procoagulante presentaban manifestaciones clínicas singulares, los pacientes se estratificaron de acuerdo a su MCF en dos grupos, uno con su MCF dentro del rango de normalidad y el otro con MCF superior al mismo. El grupo de pacientes con un MCF alto presentaba más manifestaciones clínicas, fundamentalmente a nivel pulmonar y nervioso, que aquellos con un MCF normal (tabla 21).

Manifestación clínica	Daño articular	Cutánea	Pulmonar	Cardiaca	Sistema nervioso	Daño renal	Muscular	Anti-DNA alto	Bajo C3	Bajo C4
MCF normal	80%	100%	10%	20%	10%	40%	20%	30%	30%	30%
MCF alto	100%	100%	30,8%	38,5%	38,5%	38,5%	30,7%	61,5%	42,8%	42,8%

Tabla 21. Manifestaciones clínicas observadas en LES respecto al parámetro MCF de ROTEM®. Los valores fueron considerados dentro de la normalidad en función de la media ± desviación estándar o de la mediana ± percentil 75) del grupo control, según la distribución de los datos.

5.5 CAPACIDAD TROMBOGÉNICA ASOCIADA A LAS MPS

Con el fin de determinar si el perfil procoagulante observado en pacientes con LES se debía al efecto hemostático de las MPs, se evaluó la capacidad protrombótica asociada a éstas mediante CAT. No hubo diferencias entre grupos en los parámetros evaluados con el reactivo PRP-Reagent, pero sí se observó un aumento en el ETP de pacientes con LES respecto a los controles sanos en los experimentos realizados con MP-Reagent, lo que indica un aumento de MPs ricas en FT en pacientes con LES (tabla 22).

	Controles	Pacientes con LES	p-valor
LT PRP-Reagent (min)	7,5±3,8	7,8±2,3	0,1280
Pico PRP-Reagent (nM)	90,5±40,8	86,2±52,5	0,1347
Tiempo al pico PRP-Reagent (min)	12,1±4,0	13,5±3,2	0,1489
ETP PRP-Reagent (nM/min)	1021,0±457,3	1107,0±323,0	0,6076
LT MP-Reagent (min)	13,3±3,2	13,2±2,9	0,1186
Pico MP-Reagent (nM)	194,4±76,5	224,8±66,5	0,0721
Tiempo al pico MP-Reagent (min)	16,1±3,6	16,0±3,3	0,1370
ETP MP-Reagent (nM/min)	1065,0±241,8	1188,0±313,6	0,0482*

Tabla 22. Capacidad procoagulante asociada a MPs. Los datos están expresados como media ± desviación estándar. Una $p<0,05$ se consideró significativa, y * denota diferencia significativa. Abreviaturas: LT, lag time; ETP, potencial endógeno de trombina.

También se evaluó la capacidad trombogénica del plasma mediante el reactivo PPP-Low Reagent, donde se observó una disminución en el ETP respecto al grupo control (tabla 23).

	Controles	Pacientes con LES	P-valor
LT PPPLow-Reagent (min)	4,1±0,8	4,7±1,1	0,14
Pico PPPLow-Reagent (nM)	228,7±38,82	234,6±68,7	0,93
Tiempo al pico PPPLow-Reagent (min)	7,4±0,8	7,8±1,6	0,43
ETP PPPLow-Reagent (nM/min)	1418±211,6	1236±259,1	0,026*

Tabla 23. Capacidad procoagulante del plasma. Los datos están expresados como media ± desviación estándar. Un $p<0.05$ se consideró significativo, y * denota diferencia significativa. Abreviaturas: LT, lag time; ETP, potencial endógeno de trombina.

Por otro lado, el pico obtenido en la generación de trombina asociada a MPs ricas en FT se correlacionó con la duración de la enfermedad (Fig. 34)

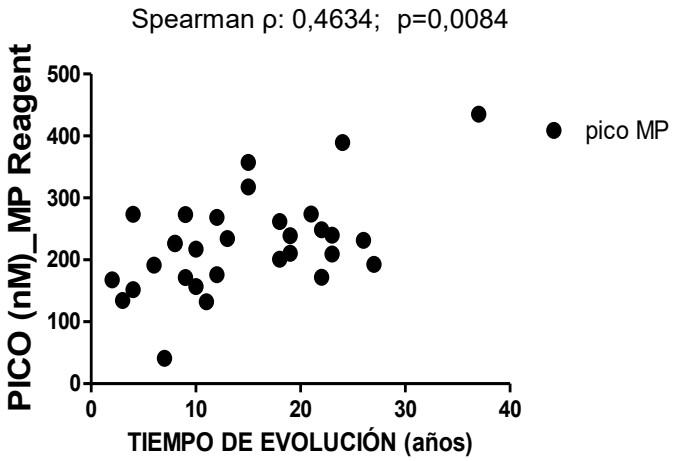


Figura 34. Correlación entre los parámetros del CAT y el tiempo de evolución de la enfermedad. El pico obtenido en la generación de trombina asociada a MPs ricas en FT correlacionó con el tiempo de evolución de la enfermedad en el grupo de pacientes. Se realizó el test de correlación de Spearman, expresado como ρ (rho) y un $p<0.05$ se consideró significativo.

5.6 EVALUACIÓN DE LA ACTIVACIÓN PLAQUETARIA

Teniendo en cuenta que las plaquetas tienen un papel esencial en la formación del coágulo, testamos su capacidad basal y su capacidad de ser activadas por agonistas.

En condiciones basales, se observó que las plaquetas de los pacientes presentaban mayor unión de PAC1, indicando que el receptor de fibrinógeno se encontraba en su conformación activa. Las plaquetas de los pacientes con LES también mostraron mayor exposición en condiciones quiescentes de P-selectina y de CD63, indicando, respectivamente, el vaciado de los gránulos-α y de los gránulos densos (Fig. 35).

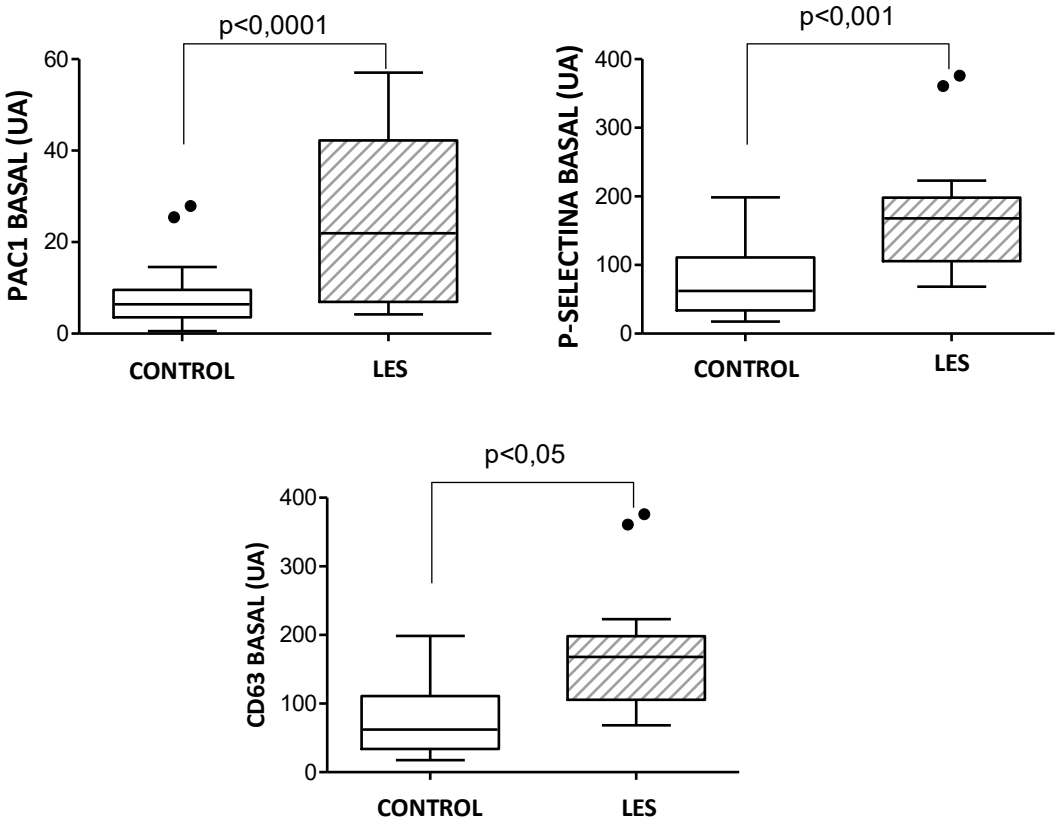


Figura 35. Actividad basal del receptor de fibrinógeno y exposición de P-selectina y CD63 en la superficie plaquetaria. El PRP de pacientes y controles fueron incubados con PAC1-FITC (A), anti-P-selectina-FITC (B) o anti-CD63-FITC y, posteriormente, analizado mediante CMF Los datos son expresados en unidades arbitrarias (AU, Fluorescencia media X % de células positivas). El análisis fue realizado con el test de Mann-Whitney y una p<0,05 se consideró significativa.

La estimulación de las plaquetas con TRAP (100 µM) o ADP (20 µM) no mostró diferencias en la unión de PAC1, ni en la exposición de P-selectina y de CD63 en la superficie plaquetaria entre ambos grupos de estudio (Fig. 36).

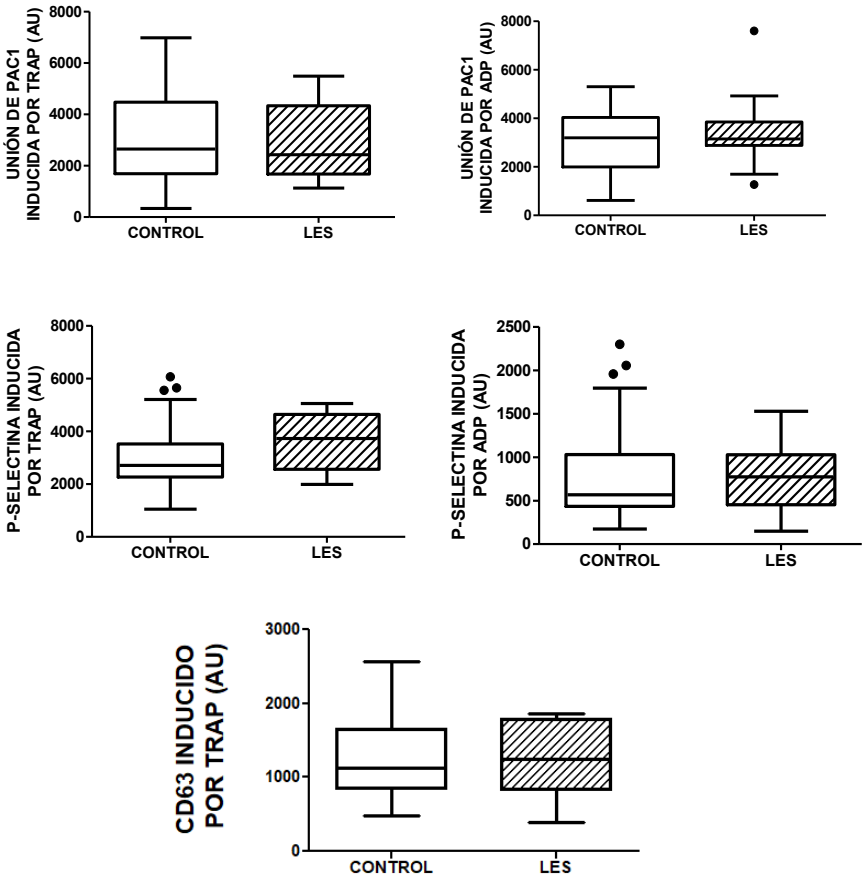


Figura 36. Actividad del receptor de fibrinógeno y exposición de P-selectina y CD63 en la superficie plaquetaria tras la estimulación con TRAP o ADP. El PRP de pacientes y controles se incubó con PAC1-FITC y ADP o TRAP, anti-P-selectina-FITC estimulado con ADP o TRAP y anti-CD63-FITC estimulado con TRAP. Las muestras se analizaron por CMF. Los datos se analizaron mediante test de Mann-Whitney y los resultados se expresaron en unidades arbitrarias (AU, Fluorescencia media X % de células positivas). No se observaron diferencias entre ambos grupos.

Es importante destacar que la activación plaquetaria en pacientes con LES fue independiente del recuento plaquetario (Tabla 24).

Recuento plaquetario (plaquetas x 103/µL) vs.	Correlación de Spearman (ρ)	P-valor
PAC basal (%)	0,1595	0,6396
P-Selectina basal (%)	-0,4	0,2229
CD63 basal (%)	-0,5714	0,1390

Tabla 24. Correlación entre el recuento plaquetario y la activación plaquetaria. La activación plaquetaria basal fue independiente del número de plaquetas. Se realizó el test de correlación de Spearman, expresado como ρ (rho) y una $p<0,05$ se consideró como significativa.

5.6.1 Expresión de las subunidades del receptor de fibrinógeno y del receptor del FvW en plaquetas

Para determinar si la activación basal observada en las plaquetas de los pacientes con LES se debía a un aumento en el número de los receptores de fibrinógeno o de FvW en su superficie, se determinó la expresión de estos receptores en ambos grupos mediante CMF. No se observaron diferencias en la expresión de las subunidades α2b (CD41b) y β3 (CD61), que forman el receptor de fibrinógeno, ni en las subunidades GPIX y GPIbα (CD42a y CD42b, respectivamente) del receptor del FvW (Fig. 37). Estos resultados indican que la activación basal de las plaquetas de los pacientes no está relacionada con la presencia de un mayor número de estos receptores en su superficie.

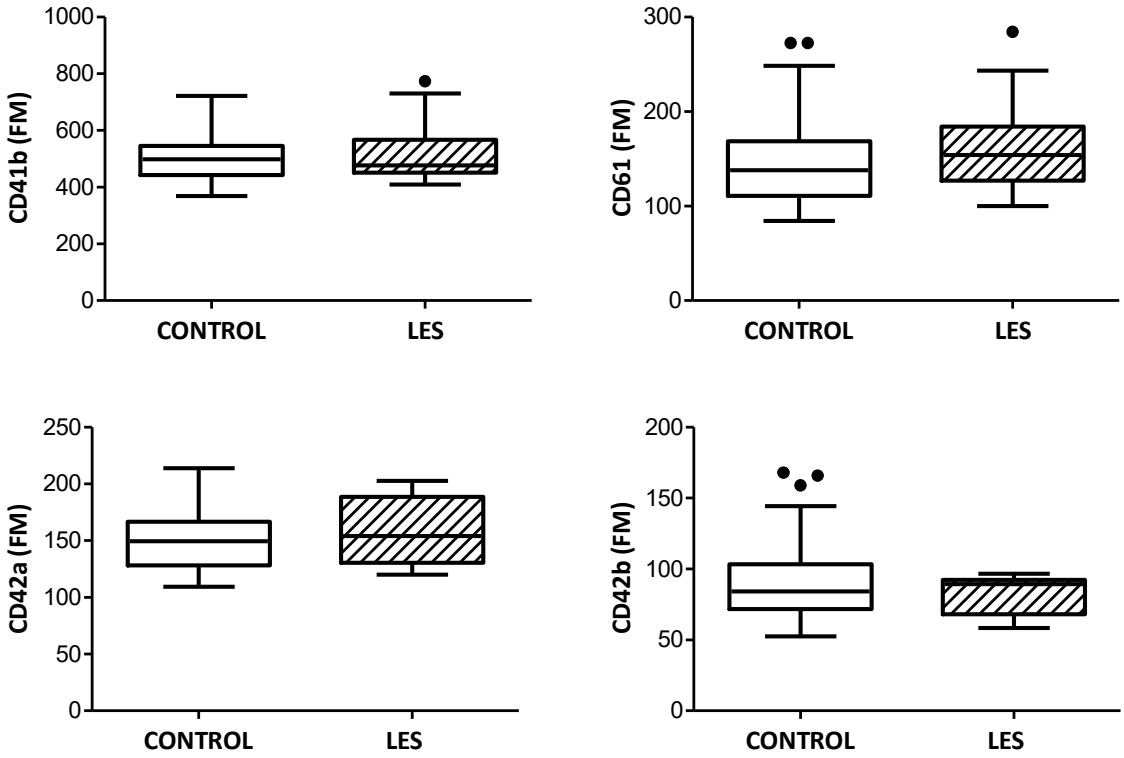


Figura 37. Determinación de las subunidades del receptor de fibrinógeno y del receptor del FvW. Para evaluar el número de subunidades del receptor de fibrinógeno y del receptor del FvW, el PRP se incubó con distintos anticuerpos y posteriormente se analizó por CMF. Los resultados están expresados como fluorescencia media (FM). La comparación entre grupos se realizó mediante el test Mann-Whitney y un valor de $p<0,05$ fue considerado significativo.

5.6.2 Estudio de la formación de agregados plaquetas-leucocitos

El estudio de la formación de agregados plaquetas-leucocitos constituye otra forma de evaluar la actividad plaquetaria. Los pacientes con LES presentaron mayor número de agregados en condiciones basales (Fig. 38). Esta diferencia entre ambos grupos desapareció cuando se evaluó la formación de agregados en condiciones estimuladas con TRAP (100 µM) o ADP (20 µM) (datos no mostrados).

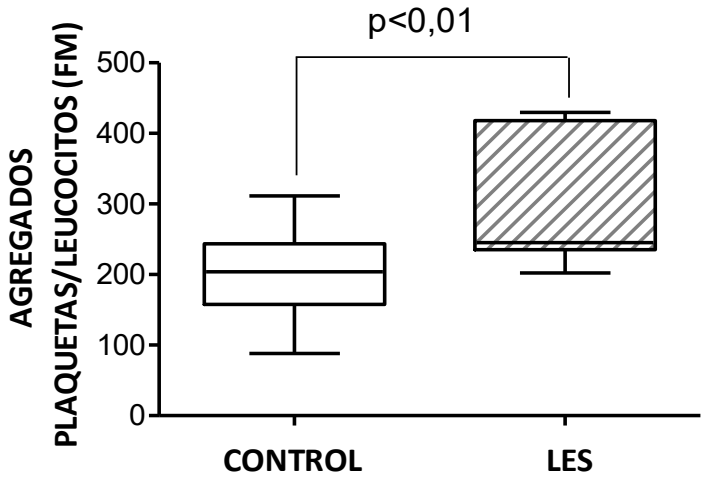


Figura 38. Formación de agregados plaquetas-leucocitos. Para evaluar la formación de agregados plaquetas-leucocitos, la sangre entera se incubó con los anticuerpos anti-CD41-PE y anti-CD45-FITC y posteriormente se analizó por CMF. Los resultados están expresados como fluorescencia media (FM) de leucocitos positivos para CD41. La comparación entre grupos se realizó mediante el test Mann-Whitney y un valor de $p<0.05$ fue considerado significativo.

El porcentaje de agregados plaquetas-leucocitos se correlacionó con los parámetros MCF (Spearman r=0,579, ρ =0,030) y con el ángulo-α (Spearman r=0,532, ρ =0,031) del ROTEM®, así como con la exposición de P-selectina sobre la superficie plaquetaria (Spearman r=0,429, ρ =0,035). Sin embargo, la formación de estos agregados fue independiente del contaje de plaquetas (Spearman r=-0,001, ρ =0,99) y de leucocitos (Spearman r=-0,23, ρ =0,44).

5.7 DETERMINACIÓN DEL GRADO DE APOPTOSIS DE LAS PLAQUETAS

Las MPs se liberan de las células cuando éstas se activan o cuando sufren apoptosis. El aumento de MPs ricas en FT observado en el CAT podría indicar no sólo una mayor activación plaquetaria sino una apoptosis exacerbada. En ambas situaciones se produce un aumento en la exposición de

PS en la superficie celular. Verificamos que las plaquetas del grupo de pacientes con LES unían más anexina V, indicando un aumento en la PS expuesta en la superficie plaquetaria (Fig.39).

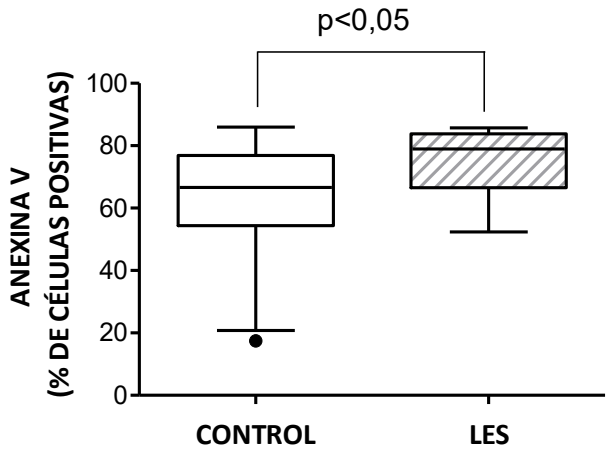


Figura 39. Exposición de PS en la superficie plaquetaria. La exposición de PS fue evaluada mediante la unión de Anexina V-FITC. Los resultados están expresados en porcentaje de células positivas. La comparación entre grupos se realizó mediante el test Mann-Whitney y un valor de p<0,05 fue considerado significativo.

5.7.1 Actividad de las caspasas plaquetarias

Para determinar si el aumento en la unión de anexina V a la PS expuesta en la superficie de las plaquetas de pacientes con LES estaba relacionada con un aumento en la apoptosis plaquetaria, se evaluó la actividad de las caspasas-3/7, -8 y -9. Sin embargo, no se observó ninguna diferencia significativa entre el grupo de pacientes con LES y el grupo control (Fig.40).

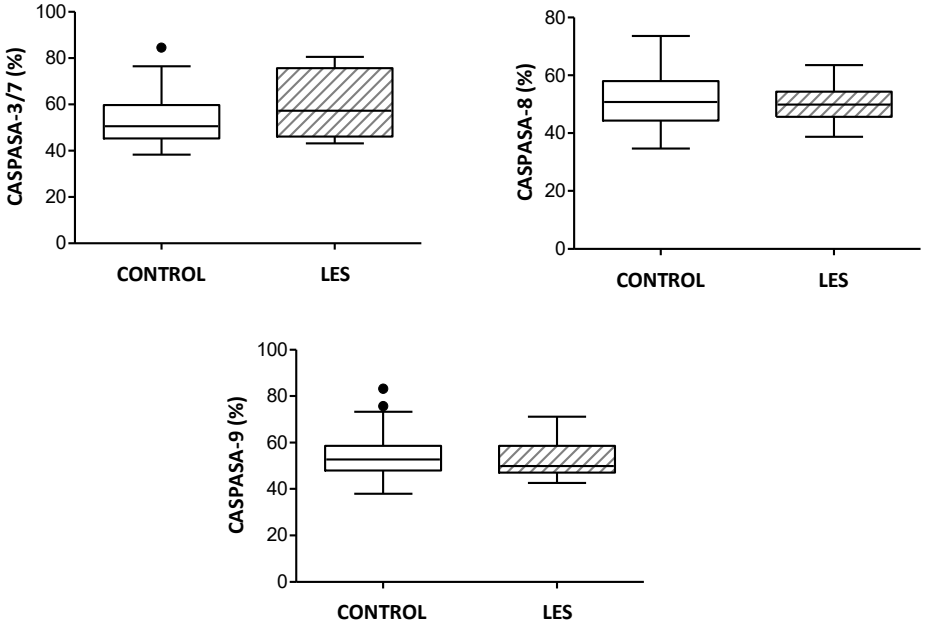


Figura 40. Actividad de las caspasas plaquetarias. Se evaluó la actividad de las caspasas-3/7, -8 y -9 por CMF. Los resultados están expresados en porcentaje. La comparación entre grupos se realizó con el test Mann-Whitney y un valor de p<0,05 fue considerado significativo.

5.8 NIVELES PLASMÁTICOS DE UN MARCADOR DE DAÑO ENDOTELIAL

El aumento de las MPs ricas en FT podía deberse al daño endotelial. Por ello se midieron los niveles plasmáticos de PAI-1, ya que se considera un buen marcador de daño de células endoteliales. Observamos que el grupo de pacientes con LES presentaban un aumento de la concentración de PAI-1 respecto al grupo control (Fig. 41).

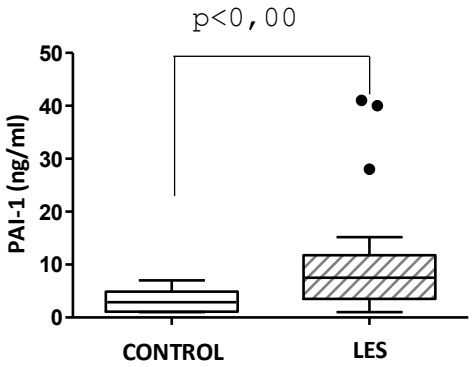


Figura 41. Niveles plasmático de PAI-1. Los niveles plasmáticos de PAI-1 se determinaron mediante un ELISA. Los datos fueron analizados mediante el test de Mann-Whitney, donde se consideró una p<0,05 como significativa.

El PAI-1 está involucrado en el control de la fibrinólisis. Observamos una correlación entre los niveles de PAI-1 con los parámetros MCF y A15 del ROTEM®, lo que sugiere una asociación entre el PAI-1 y el estado procoagulante característico de estos pacientes (Fig.42).

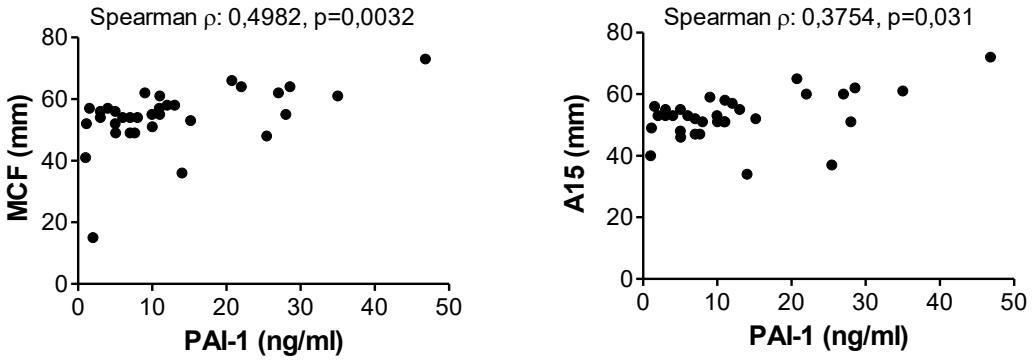


Figura 42. Correlación de los niveles de PAI-1 con los parámetros del ROTEM®. Los niveles de PAI-1 se correlacionaron con los parámetros A15 y MCF del ROTEM®. El resultado de la correlación de Spearman se muestra como ρ (rho) y una p<0,05 se consideró como significativo.

Sin embargo, los niveles plasmáticos de PAI-1 no se correlacionaron con la actividad de la enfermedad observada en pacientes (Spearman, ρ=-0,033, p=0,863).

Además, se evaluó la concentración plasmática de PAI-1 en el grupo control y en el grupo de pacientes con LES sin otras enfermedades autoinmunes, observándose un aumento de PAI-1 en este subgrupo de pacientes (Fig. 43).

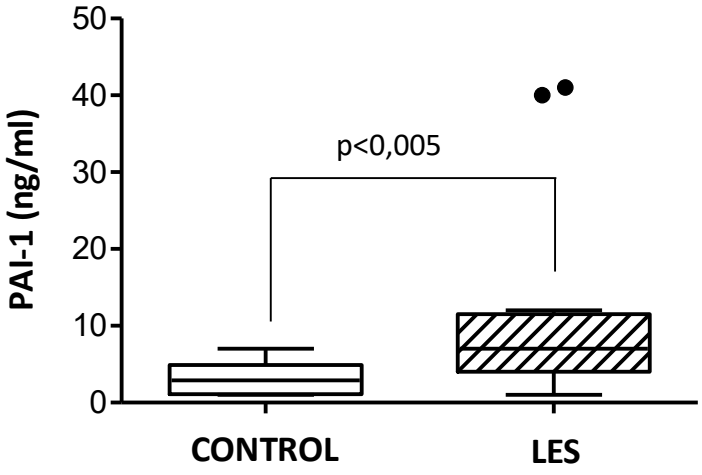


Figura 43. Niveles plasmáticos de PAI-1. El subgrupo de pacientes con LES sin otras enfermedades autoinmunes presentaron mayores niveles plasmáticos de PAI-1 respecto a los controles. Se realizó un test Mann-Whitney y se consideró significativa una p<0,05.

5.9 DETERMINACIÓN DE LOS NIVELES DE cfDNA PLASMÁTICOS Y EVALUACIÓN DE LA GENERACIÓN DE TROMBINA ASOCIADA A LAS NETS

Estudios previos han demostrado que los ácidos nucleicos contenidos en el plasma pueden dar lugar a perfiles procoagulantes. Los pacientes con LES tuvieron mayor cantidad de cfDNA en el plasma en comparación a los controles (Fig. 44 A).

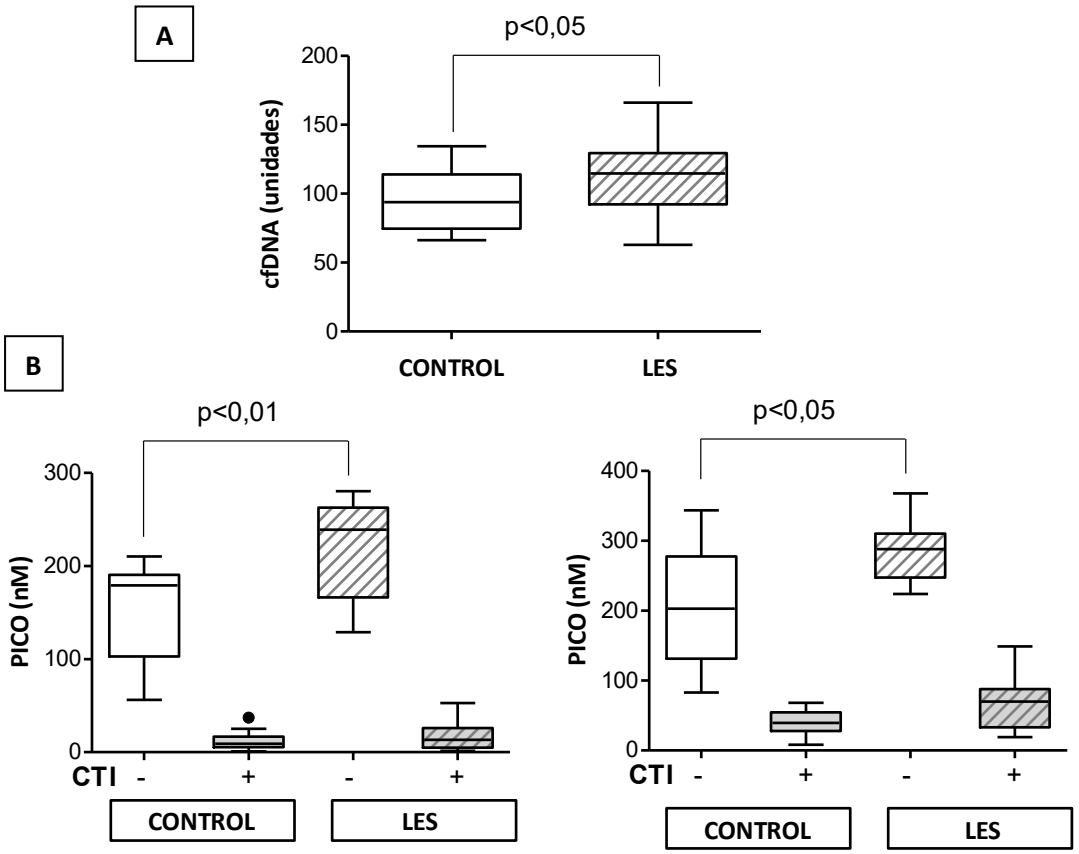


Figura 44. Generación de trombina asociada a las NETs. Se testó el efecto de las NETs en la generación de trombina en condiciones no estimuladas (A) y estimuladas con PMA 100 nM (B) en presencia de PRP de controles sanos ajustado a 10^5plaquetas/µL, con y sin CTI. Los datos fueron analizados mediante un test de Mann-Whitney y una p<0,05 se consideró significativa.

Por otro lado, la generación de trombina asociada a la presencia de neutrófilos sin estimular y estimulados para generar NETs, fue mayor en pacientes con LES. El CTI previno la generación de trombina en ambos grupos (Fig. 44 B).

Los resultados anteriores parecían indicar que los neutrófilos de los pacientes con LES liberaban más material nuclear en condiciones basales y tras estimular con PMA. Verificamos esta hipótesis utilizando microscopía de fluorescencia. La figura 45 muestra que los pacientes con LES generaban más NETs antes y después de ser estimuladas con PMA.

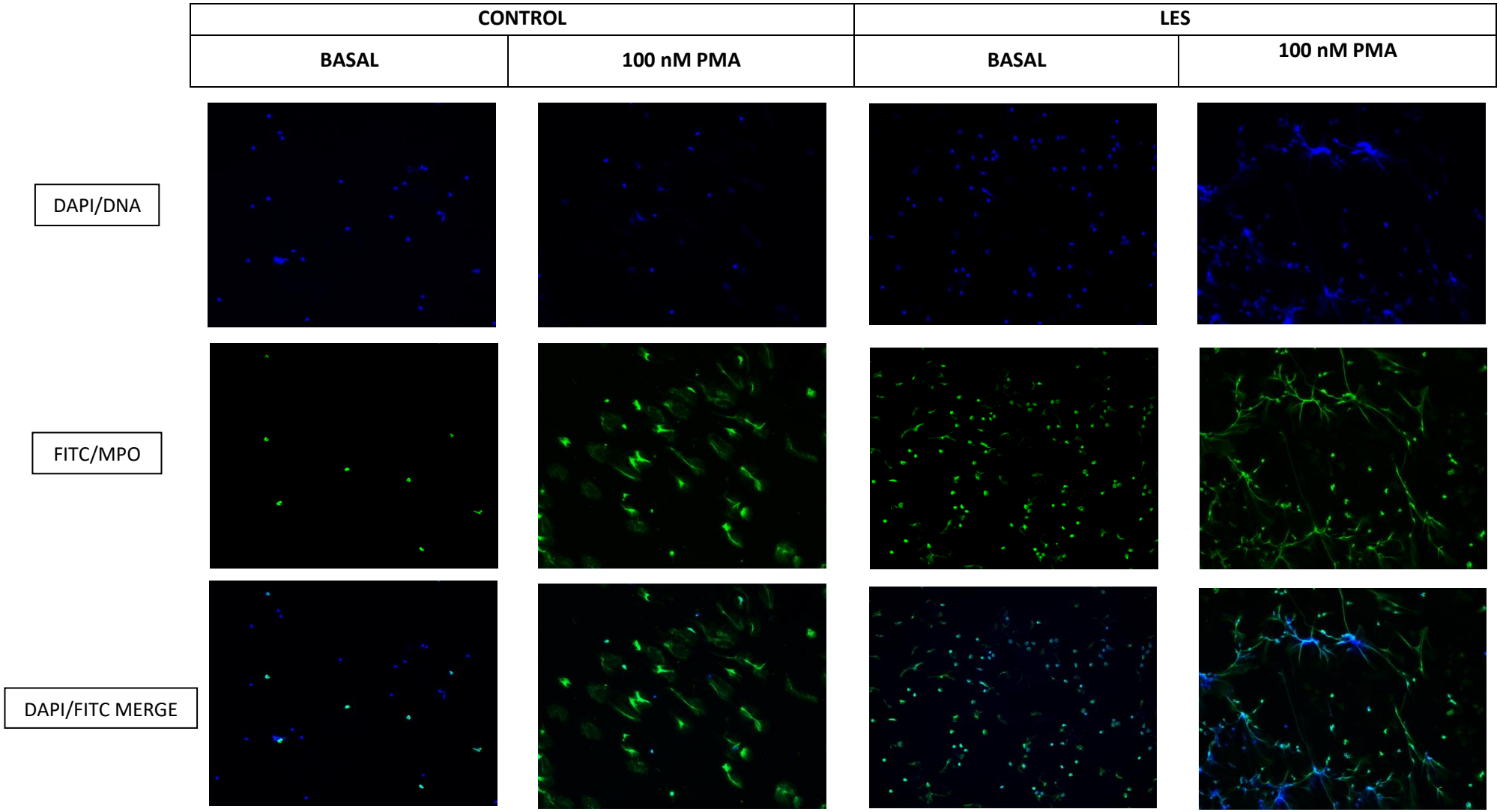


Figura 45. Imagen al microscopio de la formación de NETs. La formación de NETs en condiciones basales y tras la estimulación con PMA (100 nM) se observó por microscopía de fluorescencia. Las imágenes fueron obtenidas a 100X.

6. DISCUSIÓN

Los factores tradicionales de riesgo cardiovascular no explican completamente los altos porcentajes de eventos isquémicos observados en pacientes con LES; y los cálculos de riesgo estándar subestiman el riesgo de desarrollar la enfermedad cardiovascular. Estudios anteriores han mostrado resultados similares a los obtenidos en este trabajo: los pacientes con LES presentan activación plaquetaria basal, presentan un incremento en MPs circulantes, así como en los agregados plaquetas-leucocitos, en los niveles plasmáticos de PAI-1 y de cfDNA. Sin embargo, estos estudios se han hecho en cohortes diferentes con criterios de inclusión distintos.

En nuestro trabajo hemos evaluado todas estas variables en la misma cohorte de pacientes, detectando así relaciones entre los diferentes mecanismos involucrados en la patogénesis del LES. Destacar que los resultados obtenidos son independientes de la presencia de anticuerpos anti-FLs puesto que su presencia constituyó un criterio de exclusión.

Otro punto importante de los resultados obtenidos es la utilidad de los test globales de coagulación para estudiar la hemostasia en estos pacientes, ya que el perfil procoagulante fue detectado a pesar de la ausencia de anticuerpos anti-FLs y de eventos trombóticos previos.

Los test globales de la coagulación tienen en cuenta la relación entre todos los mecanismos involucrados (plaquetas, generación de trombina asociada a MPs, cfDNA) y deberían, si es posible, incorporarse a la práctica clínica habitual para detectar el riesgo de un evento trombótico en pacientes con LES y actuar en consecuencia, con el fin de prevenir su ocurrencia tal y como recomienda la EULAR.

6.1 CINÉTICA DE FORMACIÓN DEL COÁGULO

El análisis de la cinética de formación del coágulo utilizando el ROTEM® demostró que, pacientes con LES, sin anticuerpos anti-FLs y que carecían de antecedentes de haber sufrido eventos trombóticos, tenían un perfil procoagulante que se puso de manifiesto por el acortamiento del CT y un aumento del ángulo-α y del MCF.

Una gran cantidad de estudios han demostrado previamente que la presencia de algún tipo de anticuerpo anti-FL, entre los que se encuentran el AL, el anti-β2GP1 y la anti-cL, contribuían al desarrollo de trombosis en pacientes con LES [411, 412]. Nuestros resultados obtenidos en una cohorte de pacientes con LES sin anticuerpos anti-FLs indican que la presencia de los mismos no es la única causa que genera un estado protrombótico e incrementa el riesgo de trombosis en estos pacientes.

El ROTEM® demostró ser una técnica efectiva para evaluar la hemostasia en distintas enfermedades, así como para el diagnóstico y tratamiento de pacientes con hemorragias severas de forma rápida [413]. Sin embargo, pese a sus numerosas ventajas, existen pocos estudios en donde se haya aplicado al estudio de la hemostasia en pacientes con LES.

En nuestros experimentos activamos la coagulación con el test NATEM, que consiste en una recalcificación de la sangre entera citratada. Es un test muy sensible a cualquier cambio en el sistema de coagulación, aunque altamente inespecífico [414]. El grupo de K S Collins *y cols.* también estudió la coagulación en pacientes con LES mediante otra técnica global, la tromboelastografía (TEG), y, en contraposición a nuestros resultados, no observaron diferencias entre pacientes y controles [415]. Esta discrepancia puede estar relacionada con el activador de la coagulación empleado en cada caso. Este grupo utilizó caolín, un activador de la vía intrínseca de la coagulación, que es más potente pero menos sensible [416] y que, además, cuenta con la desventaja de que puede producir distintos artefactos [417], pudiendo ofrecer unos resultados alejados de la realidad.

Li *y cols.* (2019) evaluaron la coagulación mediante TEG en una cohorte pediátrica con LES [418] y observaron un perfil procoagulante en estos pacientes aunque, en este caso, no excluyeron a pacientes con anticuerpos anti-FLs. De forma similar, Huiyun G *y cols. r*eportaron características protrombóticas en pacientes adultos con LES y anticuerpos anti-FLs e incluso con antecedentes de eventos trombóticos [419].

Algunos estudios han propuesto que las técnicas globales como el ROTEM® y el TEG no son adecuadas para evaluar la hemostasia en el caso de individuos que presenten anticuerpos anti-FLs, ya que éstos pueden interferir en la monitorización de la coagulación y dar lugar a conclusiones erróneas [420]. Este hecho no afectaría a nuestros resultados ya que como dijimos anteriormente, la presencia de anticuerpos anti-FLs constituyó un criterio de exclusión en nuestro estudio.

Otros estudios han demostrado que síndromes autoinmunes, como el de Raynaud y el de Sjögren, inducen un perfil procoagulante [421, 422]. Parte de nuestra cohorte de pacientes mostraban estos síndromes, además de LES. Sin embargo, el efecto procoagulante de estos síndromes no parecían solaparse, puesto que no se observaron diferencias entre el grupo de pacientes con LES y el grupo de pacientes con LES y con otras enfermedades autoinmunes concomitantes.

La evaluación de las características clínicas de los pacientes con LES en función de su MCF, mostraron que, a pesar de que pacientes con un MCF alto o con un MCF dentro de la normalidad tenían un daño renal similar, aquellos con un MCF alto tuvieron aproximadamente 1,5 veces más de daño muscular y afectación cardiaca y tres veces más de manifestaciones pulmonares y del sistema nervioso, en comparación con aquellos pacientes que tenían un MCF dentro de los rangos de normalidad considerados. Aunque sería interesante evaluar estos resultados en un tamaño muestral más grande, nuestras observaciones indican la importancia de mantener a los pacientes en unos parámetros hemostáticos dentro de los rangos de normalidad. Con este fin, algunos autores proponen tratamiento profiláctico con terapia anticoagulante oral en pacientes con LES [423].

6.2 RECUENTO DE LAS SUBPOBLACIONES CELULARES

El número de plaquetas y de eritrocitos influye en las propiedades viscoelásticas del coágulo [424]. En especial, las plaquetas son responsables de la fortaleza del coágulo ya que participan en la unión de las fibras de fibrina [425]. Los pacientes con LES tenían un número de plaquetas y de eritrocitos menor que los controles, aunque dentro del rango normal. Por tanto era de esperar que estas diferencias no afectasen a los distintos parámetros del ROTEM® y, de afectarlos, hubiera resultado en un perfil hipocoagulable.

6.3 ACTIVACIÓN PLAQUETARIA Y FUNCIONALIDAD

El recuento y estado de activación de las plaquetas pueden influir en la cinética de formación del coágulo, motivo por el cual evaluamos sus características en ambos grupos.

Como marcadores del estado de activación plaquetaria evaluamos la función del receptor de fibrinógeno, esencial para una correcta hemostasia [426] y la capacidad de degranulación de los gránulos-α y de los gránulos densos. De forma similar a lo descrito por otros grupos [427], las plaquetas de los pacientes con LES de nuestra cohorte estaban activadas basalmente. Esta activación podría estar relacionada con la presencia de anticuerpos anti-dsDNA en los pacientes estudiados. Estos anticuerpos pueden aumentar la expresión de P-selectina y producir cambios morfológicos en las plaquetas, induciendo su activación. Sin embargo, esta diferencia en la activación plaquetaria desaparecía al ser estimuladas por agonistas como el TRAP o ADP.

De manera similar, Frelinger *y cols.* reportaron que las plaquetas de pacientes pediátricos con trombocitopenia inmune también presentaban un incremento en la expresión de P-selectina

en condiciones basales, pero no tras la estimulación con distintos agonistas [428]. Este hecho se podría explicar considerando que la activación basal plaquetaria puede provocar el vaciamiento de los gránulos-α o por una desensibilización de la respuesta plaquetaria a la activación con el ADP liberado de los gránulos. Esta explicación también puede justificar la mayor exposición de CD63 en la superficie de las plaquetas en estado basal pero no tras estimulación con un agonista.

Se ha descrito que la P-selectina juega un papel fundamental en el proceso inflamatorio y aterosclerótico [429], lo que convertiría a la P-selectina en un biomarcador predictivo de trombosis [430]. El hecho de que los pacientes con LES de nuestra cohorte expongan más P-selectina en la superficie de sus plaquetas a pesar de no haber padecido ningún evento trombótico podría considerarse un factor de riesgo para su ocurrencia en el futuro.

Nuestros experimentos también mostraron que los receptores de fibrinógeno unían más PAC1 en condiciones basales y este hecho no se debió a que las plaquetas de los pacientes con LES tuviesen más receptores sino al efecto de las sustancias activadoras liberadas de los gránulos en condiciones basales [431]. Este hecho podría ser responsable del aumento de la MCF del coágulo observada en el ROTEM® tal y como muestra el resultado del FIBTEM.

Es importante destacar que 18 pacientes de nuestra cohorte estaban en tratamiento con hidroxicloroquina. La hidroxicloroquina es un antipalúdico que se considera básico en el tratamiento de los pacientes con LES y que se ha visto que tiene un efecto inhibitorio sobre las plaquetas, pues inhibe la interacción del fibrinógeno con las plaquetas [432], además de inhibir la degranulación de éstas [433]. A pesar de dichos efectos demostrados en otros estudios, nosotros no encontramos diferencias en la actividad plaquetaria entre los pacientes tratados con hidroxicloroquina con los que no lo estaban (datos no mostrados).

En la última década se han publicado estudios que proponen al VMP como un marcador de activación plaquetaria, y de la actividad de la enfermedad. Estos estudios, son numerosos y sus conclusiones contradictorias [434, 435].

Un meta-análisis realizado por Chan Na Z *y cols.* concluyó, finalmente, que el VMP no puede ser empleado como un biomarcador de actividad de la enfermedad en pacientes con LES, ya que no encontraron diferencias significativas en los niveles de VMP entre pacientes con LES activo y controles sanos [436]. Nuestros resultados no mostraron correlación alguna entre la actividad de la enfermedad, evaluada por el SLEDAI-2K score, y el VMP de los pacientes.

6.4 AGREGADOS PLAQUETAS-LEUCOCITOS

Una consecuencia directa de la activación basal de las plaquetas de los pacientes con LES es el aumento en el número de agregados plaquetas-leucocitos. Ciertos estudios han demostrado que la agregación entre plaquetas y leucocitos es importante en eventos patológicos como la inflamación y la coagulación. La activación plaquetaria y la degranulación permite a las plaquetas interaccionar con los leucocitos mediante factores solubles. Esta interacción está facilitada por una gran variedad de receptores, como la αMβ2 (también conocida como Mac-1) y el PSGL-1 [437].

El aumento en la exposición de P-selectina en la superficie plaquetaria conlleva a la interacción de las plaquetas con los leucocitos, a través del receptor PSGL-1 presente en la superficie de estas células [438]. Por otro lado, la interacción entre la integrina αMβ2 de los leucocitos y la subunidad GPIb del receptor del FvW en plaquetas produce una fuerte adhesión que contribuye a los procesos trombóticos [439].

Se ha visto que las interacciones plaquetas-leucocitos facilitan el reclutamiento de leucocitos y su extravasación al lugar de la inflamación, promueve la liberación de mediadores proinflamatorios amplificando la respuesta celular proinflamatoria [440] y el estrés oxidativo, así como la fagocitosis y la liberación de NETs.

Por tanto, el aumento de formación de agregados en condiciones basales observado en pacientes con LES respecto a los controles estaría favoreciendo el estado inflamatorio crónico en estos pacientes.

6.5 EXPOSICIÓN DE PS EN LA SUPERFICIE DE LAS PLAQUETAS Y ACTIVIDAD DE LAS CASPASAS EN LOS PACIENTES CON LES

La activación plaquetaria produce una mayor exposición de PS en la superficie plaquetaria [441]. Nuestros resultados mostraron que las plaquetas de los pacientes con LES exponían más PS en su superficie en comparación a las plaquetas procedentes de los controles sanos. El aumento en la superficie de PS contribuiría al perfil protrombótico [442] de los pacientes con LES ya que proporciona una superficie de anclaje para el complejo protrombinasa y tenasa, promoviendo la generación de trombina [443] y aumentando la efectividad de la coagulación sanguínea [444]. Trabajos previos de nuestro laboratorio demostraron que las plaquetas de los pacientes con trombocitopenia inmune [445] y con síndromes mielodisplásicos [446], exponían

más PS y unían más complejo protrombinasa (FVa y FXa), lo que confirma que el aumento de la PS en la superficie de las plaquetas es funcional para el anclaje del complejo protrombinasa.

Otra situación que puede provocar el aumento de exposición de PS en la superficie celular es la apoptosis. Se ha descrito que la exposición de PS sobre la superficie de las plaquetas actúa como una señal "eat-me" implicada en la eliminación de estas células [447]. Con el objeto de determinar si el aumento en la exposición de PS en las plaquetas de los pacientes con LES podía deberse no sólo a la activación basal sino también a la apoptosis, determinamos la actividad de las caspasas-3/7, 8 y 9 y no hallamos diferencias con el grupo control. Esto apoya la hipótesis de que el incremento de la exposición de PS observada en las plaquetas de nuestro grupo de pacientes se debe a la activación basal encontrada en ellas y no a la exacerbación de un proceso apoptótico o necrótico.

6.6 CAPACIDAD PROTROMBÓTICA ASOCIADA A MPs

La sangre contiene MPs, que son liberadas por activación o apoptosis celular [448]. Pueden provenir de plaquetas, monocitos y células endoteliales. Independientemente de su origen, todas tienen características protrombóticas, ya que proporcionan una superficie de PS donde se anclan distintas proteasas de la cascada de coagulación.

Se ha descrito que la sangre de los pacientes con LES contienen más MPs, favoreciendo así la inflamación crónica y el daño tisular característicos de esta patología [449-451]. Dieker *y cols.* demostraron que las MPs de pacientes con LES tienen un fuerte efecto proinflamatorio sobre las células dendríticas [452]. Además, también se ha demostrado que las MPs tienen efecto sobre los granulocitos, haciendo que estos inicien el proceso de degranulación y la producción de especies reactivas de oxígeno, favoreciendo la generación y liberación de NETs [453].

Los resultados obtenidos en el CAT mostraron una mayor generación de trombina asociada al contenido de FT de las MPs, pero no al contenido de PS de las mismas. Mobarrez *y cols.* demostraron que las MPs de pacientes con LES eran PS-negativas [454], aunque el grupo de Pereira *y cols.* observó una mayor generación de trombina dependiente de PS [449]. Esta última observación coincide con el hecho de que Sellam *y cols.* reportaran un aumento en MPs ricas en PS [455].

Las discrepancias entre estos estudios se pueden deber a los distintos criterios de inclusión de los pacientes y/o a las distintas técnicas empleadas para medir MPs. Dado que las MPs en LES muestran características moleculares y fenotípicas únicas incluyendo la extraña expresión de

PS, se ha discutido mucho sobre la utilidad de éstas como biomarcador, así como el papel de las MPs en la patogénesis del LES [451].

Las MPs que proceden de monocitos y células endoteliales son ricas en FT, mientras que las de plaquetas son trombogénicas por la presencia de receptores para los ligandos de adhesión [456] y PS [457].

El hecho de que en los pacientes con LES encontrásemos un aumento en el potencial trombogénico de MPs ricas en FT, sugiere un origen endotelial.

La correlación observada en el grupo de pacientes entre el perfil procoagulante y la duración de la enfermedad, pero no con su índice de actividad, sugiere que el daño crónico parece influir más que la actividad de la enfermedad en el estado hipercoagulable de estos pacientes. Sin embargo, no se puede descartar que la falta de correlación entre los parámetros de coagulación y la actividad de la enfermedad podría deberse al tamaño muestral de nuestra cohorte de estudio.

6.7 DAÑO ENDOTELIAL Y ACTIVIDAD DE LA ENFERMEDAD

Se ha descrito que el endotelio juega un papel fundamental en la patofisiología del LES, donde se ha demostrado un elevado número de células endoteliales en circulación, apoptosis y activación del endotelio [458], lo que produce aterosclerosis diseminada, que es el estado preclínico del desarrollo de la enfermedad cardiovascular temprana.

La disfunción endotelial es el resultado del daño que sufren las células endoteliales que no pueden mantener un equilibrio normal entre la vasodilatación y la vasoconstricción, la formación del coágulo y la fibrinolisis, así como la proliferación y migración de células del músculo liso [459].

Como el incremento de MPs ricas en FT parece estar asociado al daño endotelial [460] y ya que el LES se caracteriza por una disfunción de éste, decidimos evaluar el grado de disfunción endotelial existente en nuestra cohorte a estudio. El PAI-1, que se encuentra en células endoteliales y en plaquetas, es considerado como un marcador de daño endotelial [461].

De acuerdo con lo referido por otros autores [462], nuestros resultados reflejaron un aumento de los niveles plasmáticos de PAI-1 en pacientes con LES respecto al grupo control.

Los niveles elevados de PAI-1 podrían estar involucrados en un aumento de la fortaleza del coágulo que observamos con el ROTEM®. También se ha descrito que el PAI-1 favorece el

aumento en la velocidad de formación del coágulo [463, 464] al inhibir, de forma irreversible, al tPA [465]. Los datos obtenidos concuerdan con esta hipótesis ya que los niveles de PAI-1 correlacionaron con los parámetros MCF y A15 del ROTEM®.

En este trabajo también determinamos los niveles de E-selectina y de LDL-oxidada como marcadores de daño y/o activación del endotelio. La LDL-oxidada es fundamental en el inicio y progresión del proceso aterosclerótico ya que participa en la inflamación crónica, en la acumulación de lípidos y en la modificación de células de la pared vascular. Se ha descrito que la LDL-oxidada puede formar ICs con las glicoproteínas β_2GPI y anti-β_2GPI, promoviendo la fagocitosis en los macrófagos y favoreciendo la formación de células espumosas en la pared vascular, ayudando a originar eventos ateroscleróticos [466, 467].

Por otro lado, el estudio de la E-selectina es muy interesante ya que se diferencia de otras moléculas de adhesión en que solamente se encuentra en el endotelio activado y se libera en respuesta a citoquinas proinflamatorias [468] y se ha considerado como un marcador de aterosclerosis [469]. La E-selectina media la adhesión de los leucocitos circulantes al endotelio vascular, paso esencial para la extravasación de estos durante en proceso inflamatorio.

Por tanto, los niveles elevados de LDL-oxidada y de E-selectina observados en nuestros pacientes y reportados por otros autores [470-472] indican la existencia de daño en el endotelio vascular que es característica del LES.

En varios estudios se ha observado una correlación del índice SLEDAI con los niveles de las proteínas del complemento C3, C4 [473] y el perfil procoagulante en estos pacientes. Sin embargo nosotros y otros autores [474, 475] no observamos esa correlación. Esto puede deberse a que los niveles de complemento pueden variar por distintos factores y no es necesariamente representativo de la actividad clínica del LES [476].

El objetivo del tratamiento en estos pacientes es mantener la remisión o disminuir la actividad de la enfermedad, así como evitar nuevos brotes. Nuestra cohorte de pacientes con LES estaban tratados de acuerdo a las recomendaciones de la EULAR [477] , encontrándose en remisión aquellos que no tenían tratamiento en el momento del estudio. Sin embargo, debido a la complejidad y diversidad de la patogénesis de esta enfermedad, la mayor parte de los pacientes necesitan una terapia combinada que puede incluir inmunosupresores (corticoesteroides, azatioprina, metotrexato, ciclofosfamida y micofenolato) y anticuerpos monoclonales bloqueantes de CD20 (rituximab) o de factores activadores de células B (belimumab). En particular, uno de los pacientes incluidos en el estudio recibió rituximab y

belimumab al mismo tiempo. Se ha demostrado recientemente que esta terapia combinada reduce la inflamación mediada por ICs, así como la formación de NETs [478].

6.8 PROCESO DE NETosis EN PACIENTES CON LES

Nuestros resultados mostraron que los neutrófilos de los pacientes con LES producían más NETs de forma espontánea y tras estimulación que los de controles sanos.

El daño de las células endoteliales en el LES podría deberse, al menos en parte, a la presencia de niveles elevados de un subconjunto de neutrófilos patógenos, los LDNs, que contribuyen a la patogénesis del lupus a través de respuestas proinflamatorias elevadas, capacidad fagocítica alterada, y daño vascular [112]. Además, este subconjunto de neutrófilos tiende a liberar más NETs de tipo red [479, 480] que están compuestos de DNA, histonas, proteínas antimicrobianas, fibrinógeno, FXII y FT [480, 481]. Este hecho podría explica el aumento de los niveles de DNA encontrado en el plasma de pacientes con LES. Además, la mayoría de los pacientes con LES tienen una capacidad reducida para degradar las NETs [479], y la presencia prolongada de NETs en el plasma podría promover la ruptura de la tolerancia inmune y aumentar el daño tisular [482]. Por lo tanto, los componentes de las NETs podrían inducir la formación de autoanticuerpos, los que a su vez formarían complejos inmunes generando un circuito amplificador del proceso inmunoinflamatorio [482]. De esta forma se perpetuaría la formación de NETs y el desequilibrio hemostático en estos pacientes.

El grupo de Etulain *y cols.* demostró en ratones que la presencia de P-selectina y PSGL-1, inducía un aumento en la formación de NETs [483]. La activación plaquetaria basal con la consecuente exposición de P-selectina podría explicar por qué los neutrófilos de los pacientes con LES fueron más susceptibles de generar NETs. Por otra parte se ha visto que las NETs están asociadas con la formación de la placa coronaria y la desregulación lipoproteica [484], manifestación clínica que se observa en muchos pacientes con LES [485].

Nuestros resultados mostraron que las NETs aumentaban la capacidad trombogénica en los pacientes con LES y que ésta estaba asociada a la vía intrínseca de la coagulación. En apoyo a esta observación, y de acuerdo con otros grupos, la generación de trombina asociada a las NETs se inhibió en presencia de CTI, un inhibidor del FXIIa y, por tanto, de la fase de contacto de la coagulación [486].

6.9 LIMITACIONES DEL ESTUDIO

- Algunos datos se recogieron de forma retrospectiva a partir de las historias clínicas de cada paciente, por lo que alguno de ellos estaban incompletos.

- La falta de pacientes con distintos tratamientos impidieron la realización de un subestudio para evaluar en profundidad el efecto de los estos en la hemostasia de los pacientes con LES.

- Aunque el tamaño muestral fue relativamente pequeño, los análisis realizados fueron suficientemente potentes, lo que hace poco probable que las diferencias observadas entre el grupo control y el grupo de pacientes sean el resultado de un sesgo significativo de reclutamiento.

- Se trata de un estudio transversal, donde los análisis estadísticos se basaron en la determinación de cada variable en un momento dado, por lo tanto, no se pudieron determinar los cambios de los diferentes parámetros evaluados a lo largo del tiempo.

7. CONCLUSIONES

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En los pacientes con LES sin anticuerpos anti-FLs ni signos de trombosis existe un perfil procoagulante que es independiente de los factores de riesgo clásicos y de la presencia de otras enfermedades autoinmunes concomitantes.

- El mecanismo etiopatogénico del LES es la suma de un funcionamiento inadecuado de distintos procesos debido a una compleja desregulación del sistema inmune en estos pacientes. En los pacientes con LES suele haber una activación basal plaquetaria que provoca un aumento en la exposición de PS en su superficie que propiciaría la unión del complejo tenasa y protrombinasa, favoreciendo la formación del coágulo.

- La activación plaquetaria basal observada en los pacientes con LES provocó un aumento en la exposición de P-selectina que causó una mayor interacción entre las plaquetas y los leucocitos formando agregados que participarían en la disfunción del endotelio vascular.

- Los pacientes con LES presentaron una disfunción del endotelio vascular, que se manifestó por un aumento en los niveles de E-selectina, de LDL-oxidada y de PAI-1, lo que contribuiría al estado procoagulante observado en ellos.

- La activación/daño del endotelio vascular causó, de manera dependiente con la duración de la enfermedad, la liberación de MPs ricas en FT que provocaron un aumento en la generación de trombina.

- El daño endotelial sería el responsable del aumento del cfDNA observado en nuestra cohorte de pacientes con LES.

- La desregulación inmunológica y el daño tisular presente en los pacientes con LES podrían ser los inductores del aumento de la actividad de los neutrófilos y la formación de NETs. Las NETs tendrían un papel importante en el perfil procoagulante de estos pacientes.

- Los test globales pueden ser considerados como herramientas útiles para evaluar la hemostasia en pacientes con LES y su uso debería ser incorporado, en la medida de lo posible, en la práctica clínica habitual como una aproximación médica orientada al paciente con el fin de prevenir posibles eventos trombóticos.

8. REFERENCIAS

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autoimmune anti-glutamate receptor antibodies can bind neurons in few brain regions, activate glutamate receptors, decrease glutamate receptor's expression, impair glutamate-induced signaling and function, activate blood brain barrier endothelial cells, kill neurons, damage the brain, induce behavioral/psychiatric/cognitive abnormalities and ataxia in animal models, and can be removed or silenced in some patients by immunotherapy. J Neural Transm (Vienna), 2014. **121**(8): p. 1029-75.

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9. ANEXOS

9.1 Premios

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La FUNDACIÓN ESPAÑOLA DE TROMBOSIS Y HEMOSTASIA

entrega el **Premio para Formación en Hemostasia y Trombosis**

(Beca Modalidad A)

Al proyecto titulado:

"Evaluación del estado procoagulante, la desregulación inmunológica

y la comorbilidad cardiovascular asociada en pacientes con lupus eritematoso sistémico"

De la investigadora:

Elena Monzón Manzano

Dear Elena Monzon Manzano,

I am delighted to inform you that you have been selected to receive an ASH Abstract Achievement Award for your abstract titled Thrombin Generation Related to Netosis in Patients with Systemic Lupus Erythematosus (#141192). The ASH Abstract Achievement Award is a merit-based award for trainees who are the first author and presenter on a high-scoring annual meeting abstract. ASH encourages you to include this honor in your curriculum vitae.

Due to the virtual nature of this year's meeting, award recipients will receive a complimentary "Real-Time Experience" registration to the 62nd ASH Annual Meeting and Exposition, taking place virtually December 5-8, 2020, in lieu of a check to cover travel expenses. This registration provides access during peak dates and hours of the meeting with the largest "live" audience at a given time, maximizing opportunities for interactions, networking, and earning CME/MOC credits, which are only available for attending live sessions.

Congratulations on your achievement! Please see further information here and a listing of your fellow recipients here. We look forward to seeing you at the virtual meeting, and thank you for your dedication and contributions to advancing the field of hematology.

Sincerely,
Patricia Frustace
Director of ASH Awards and Diversity Programs

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De la investigadora:

Elena Monzón Manzano

CÂMARA MUNICIPAL DE GUIMARÃES

Prémio Príncipe da Beira
Ciências Biomédicas 2017

3ª classificada de Excelência atribuída a

Maria Elena Monzón Manzano

com o trabalho intitulado *Evaluation of hypercoagulate State, Immunological Dysregulation and Comorbidity Related to Cardiovascular Disease in Patients with Systemic Lupus Erythematosus.*

Guimarães, 20 de Abril de 2018

Presidente da Câmara Municipal de Guimarães

Dr. Domingos Bragança

Vice-Reitor para a Investigação da Universidade do Minho

Prof. Doutor Rui Reis

Presidente da Fundação Dom Manuel II

SAR Dom Duarte, Duque de Bragança

9.2. Trabajos presentados a congresos

331.PATHOPHYSIOLOGY OF THROMBOSIS | NOVEMBER 5, 2020

Thrombin Generation Related to Netosis in Patients with Systemic Lupus Erythematosus

Elena Monzón Manzano, Ihosvany Fernandez-Bello, BSPharm,PhD, Raul Justo Sanz, Ángel Robles Marhuenda, Paula Acuña, María Teresa Alvarez Román, MD PhD, Elena G Arias-Salgado, PhD, Sara García Barcenilla, Miguel A Canales, MD PhD, Victor Jimenez-Yuste, MDcal Degree. PhD, Nora V. Butta, PhD

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NETosis is a process suffered by neutrophils that consists in the loss of their function and the release of their nuclear material as large web-like structure called neutrophil extracelular traps (NETs). Many authors demonstrated that NETs participate in the pathogenesis of autoimmune diseases, such as systemic lupus erythematosus (SLE), because the release of autoantigens amplifies inflammatory responses, perpetuating the exacerbation of autoimmunity. On the other hand, NETs may play a prominent role in thrombosis because they serve as a negative charge scaffold to trap platelets and coagulation factors, promoting blood clot formation.

Objetive: to determine participation of NETs in the hypercoagulable state of patients with SLE.

Methods: 32 patients with SLE without antiphospholipid antibodies and without history of thrombotic events were included after signing informed consent; 88 sex- and age-matched healthy controls were also recruited. Blood samples were drawn in citrate tubes (3.2%). Neutrophils were isolated by centrifugation of whole blood with a Percoll gradient at 500 g, 25 min, 5ºC. To induce NETs formation, $2.5x10^5$ isolated neutrophils were incubated in RPMI-1640 medium with or without 100 nM phorbol 12-myristate 13-acetate (PMA) for 45 min, 37ºC.

To verify NETs formation, neutrophils were seeded on cover glasses pretreated with poly-L-lysine in RPMI-1640 medium with or without 100 nM PMA for 45 min, 37ºC. Samples were fixed and later incubated first, with an anti-human myeloperoxidase and then, with Alexa Fluor 488 goat anti-rabbit IgG. Finally, samples were embedded in mounting medium with DAPI and were observed by fluorescence microscopy with a Nikon Eclipse 90i microscope.

Cell free DNA (cfDNA) was determined in poor platelet plasma obtained by centrifugation of whole blood (2500 g for 15 min), using the Quant-iT™ Pico Green dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

To assess thrombin generation associated to NETs, $2.5x10^5$ neutrophils from patients with SLE or from controls were incubated with either buffer or 100 nM PMA during 45 min. Then they were centrifuged at 5000g, 3 min and resuspended in 40-µL of rich platelet rich plasma (PRP) from healthy controls adjusted to 10^6 platelets/µL obtained from blood samples drawn either in citrate or citrate plus corn trypsin inhibitor (CTI) tubes. CTI is an inhibitor of FXIIa. Calibrated automated thrombogram (CAT) was performed without addition of any trigger.

Results: We observed that plasma from patients with SLE had increased free nucleic acids (cfDNA in fluorescence units, controls: 94.90±21.29, SLE patients: 112.4±26.59; P=0.0211). In accordance with this observation, analyses by fluorescence microscopy showed that neutrophils from SLE patients, but not from controls, had NETs even in basal conditions. Moreover, neutrophils from these patients generated more NETs in presence of 100 nM PMA (Figure 1).

To evaluate whether the increment of NETs observed in patients with SLE had consequences on the hemostasis of these patients, we tested thrombin generation of neutrophils from either patients with SLE or controls in the presence of platelets from healthy controls. Neutrophils from patients with SLE produced more thrombin than those from healthy controls under basal conditions and after stimulation with 100 nM PMA. These increments were avoided when PRP was collected from blood samples drawn with CTI (Figure 2).

Conclusions: Neutrophils from SLE patients without antiphospholipid antibodies and with no history of thrombotic seemed more prone to form NETs than those from healthy controls. NETs might be considered as a key element in the prothrombotic profile of patients with SLE and their analyses by thrombin generation test might be useful to detect risk of occurrence of thrombotic events in these patients and to prevent its occurrence by therapeutic management.

This work was supported by grants from FIS-FONDOS FEDER (PI19/00772).EMM holds a predoctoral fellowship from Fundación Española de Trombosis y Hemostasia (FETH-SETH).

Figure 1. Formation of NETs. NETs were evaluated after stimulation with 100 nM PMA. DNA (DAPI, blue), neutrophil myeloperoxidase (MPO, FITC, green) and DAPI/FITC merge images are shown. Original magnification x10.

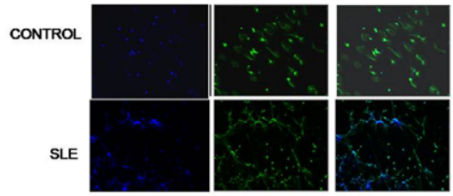
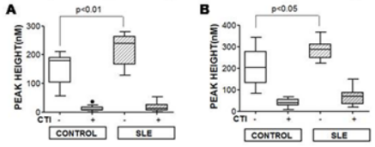


Figure 2. Thrombin generation associated with NETs. The effect of NETs on thrombin generation was tested in either non stimulated (A) or 100 nM PMA-stimulated neutrophils (B) in the presence of PRP from healthy controls adjusted to $1x10^5$ platelets/µL, with (+) or without (-) CTI. A Mann–Whitney test was performed, and $P<0.05$ was considered significant.



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Fernandez-Bello:*Stago:* Speakers Bureau; *Pfizer:* Speakers Bureau; *SOBI,:* Research Funding; *Roche:* Speakers Bureau; *Novartis:* Speakers Bureau; *Takeda:* Research Funding, Speakers Bureau; *NovoNordisk:* Current Employment, Research Funding, Speakers Bureau. **Justo Sanz:***Takeda:* Current Employment. **Alvarez Román:***Bayer:* Consultancy; *Grifols:* Research Funding; *Pfizer,:* Research Funding, Speakers Bureau; *SOBI,:* Consultancy, Research Funding, Speakers Bureau; *Takeda:* Research Funding, Speakers Bureau; *NovoNordisk,:* Research Funding, Speakers Bureau; *Roche:* Speakers Bureau; *Novartis:* Speakers Bureau. **García Barcenilla:***Novartis:* Speakers Bureau; *Roche:* Speakers Bureau; *Pfizer,:* Speakers Bureau; *NovoNordisk:* Research Funding, Speakers Bureau; *Takeda:* Research Funding, Speakers Bureau; *Bayer:* Speakers Bureau. **Canales:***Sandoz:* Speakers Bureau; *Roche:* Honoraria; *Sandoz:* Honoraria; *Karyopharm:* Honoraria; *Roche:* Speakers Bureau; *Takeda:* Speakers Bureau; *Roche:* Honoraria; *Takeda:* Speakers Bureau; *Novartis:* Honoraria; *Sandoz:* Speakers Bureau; *Karyopharm:* Honoraria; *Roche:* Speakers Bureau; *Janssen:* Honoraria; *Janssen:* Speakers Bureau; *iQone:* Honoraria; *Sandoz:* Honoraria; *Gilead:* Honoraria; *Janssen:* Speakers Bureau; *Celgene:* Honoraria; *Janssen:* Honoraria; *Novartis:* Honoraria. **Jimenez-Yuste:***F. Hoffman-La Roche Ltd, Novo Nordisk, Takeda, Sobi, Pfizer:* Consultancy; *F. Hoffman-La Roche Ltd, Novo Nordisk, Takeda, Sobi, Pfizer, Grifols, Octapharma, CSL Behring, Bayer:* Honoraria; *Grifols, Novo Nordisk, Takeda, Sobi, Pfizer:* Research Funding. **Butta:***Novartis:* Speakers Bureau; *NovoNordisk:* Speakers Bureau; *Takeda:* Research Funding, Speakers Bureau; *SOBI:* Speakers Bureau; *Grifols:* Research Funding; *ROCHE:* Research Funding, Speakers Bureau; *Pfizer:* Speakers Bureau.

Author notes

* Asterisk with author names denotes non-ASH members.

Characterization of the Hypercoagulable State and NETosis in Systemic Lupus Erythematosus

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Abstract Number: PB1644

Meeting: ISTH 2020 Congress

Theme: Platelets and Megakaryocytes » Platelet Function and Interactions

Background: Many mechanisms are involved in thrombotic processes that contribute to morbidity in patients with systemic lupus erythematosus (SLE). Global coagulation tests may be valuable to evaluate SLE hypercoagulable state.

Aims: To characterize the prothrombotic state by Rotational Thromboelastometry (ROTEM®) and Calibrated Automated Thrombogram (CAT®) in SLE not mediated by antiphospholipid antibodies (aPL).

Methods: 32 patients with SLE without aFL and 88 healthy controls were recruited after signing the informed consent. Experimental protocol was approved by La Paz University Hospital Ethics Committee. Kinetic clot formation was determined by ROTEM®. Platelet activation markers, exposure of phosphatidylserine (PS) and caspase activities were determined by flow cytometry. To generate neutrophil extracellular traps (NETs) neutrophils from controls or SLE patients were stimulated with 100 nM PMA and cocultivated with PRP from healthy controls adjusted to 10^5 platelets/µL with/without corn trypsin inhibitor (CTI) to block contact phase. Thrombin generation associated to NETs and to microparticle's (MPs) content of tissue factor (TF) was measured by CAT. Plasma levels of cfDNA and of PAI-1 were also determined. Statistical analysis was performed by Prism-Graphpad.

Results: ROTEM® showed a procoagulant profile in SLE patients (Fig 1 A). Plasma PAI-1 was increased in SLE patients (Fig.1B) and its levels correlated with several ROTEM parameters (Fig. 1C). SLE patients showed a basal platelet activation and a higher PS exposure (Fig.1D) that was not due to an enhanced apoptosis and an increased thrombin generation associated to TF content of MPs, to cfDNA and to NETs (Fig.2). cfDNA- and NETs- associated thrombin generation were prevented by CTI.

Conclusions: ROTEM® detected a hypercoagulable state in SLE patients that might be linked to increased plasma PAI-1, basal platelet activation with a consequent enhanced exposure of PS, augmented TF-containing MPs and cfDNA. Moreover, neutrophils from SLE patients seemed more prone to form NETs than those from healthy controls.

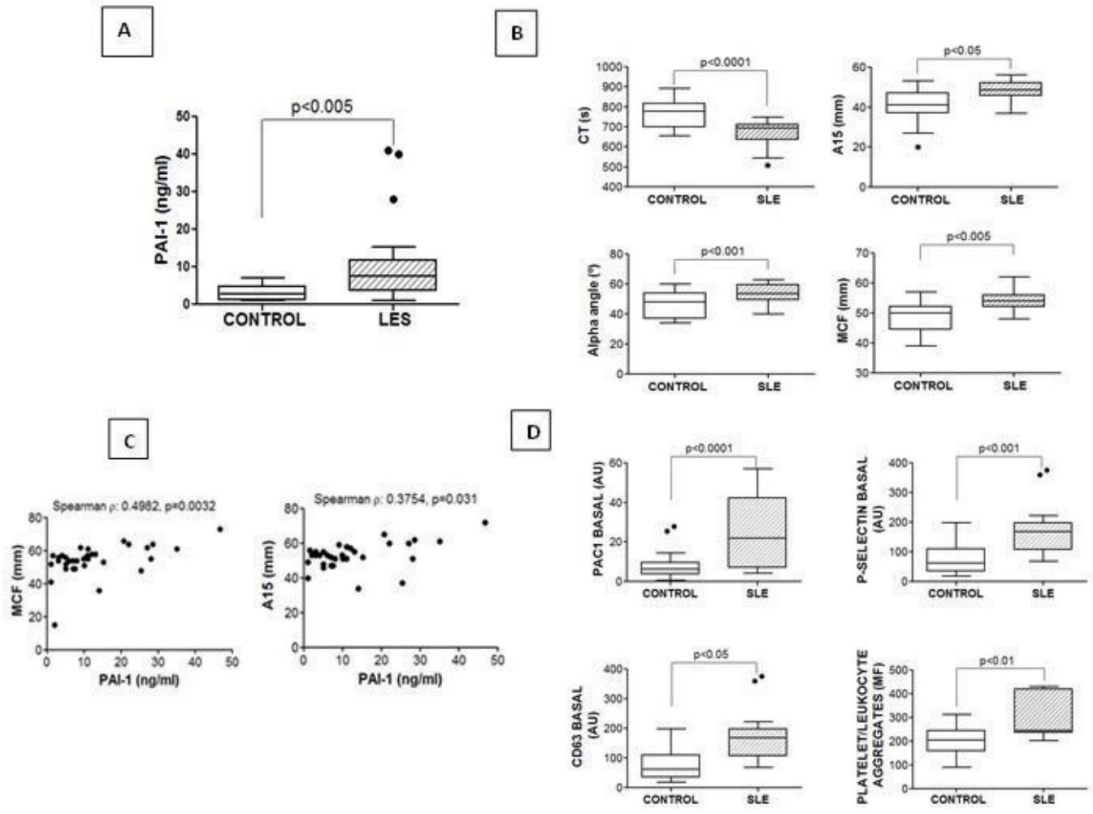

Fig. 1. Procoagulant profile in patients with SLE. A) PAI-1 plasma level was increased in SLE patients. **B)** ROTEM was performed in whole blood. SLE, systemic lupus erythematosus; CT, clotting time; A15, amplitude at 15 min; MCF, maximum clot firmness. **C)** Correlation between PAI-1 plasma levels with ROTEM parameters. **D)** Basal activity of fibrinogen receptor, surface exposition of P-selectin, CD63 and PS (determined by annexin V binding). Data are expressed as arbitrary units (mean fluorescence X % of positive cells) or only as % of positive cells. p≤0.05 was stablished as statistical significance.

[Figure 1]

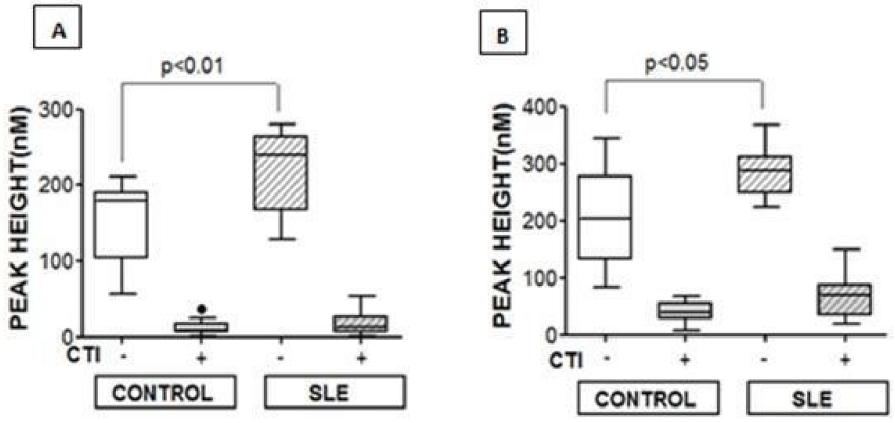

Fig. 2. Thrombin generation associated to NETs. **A)** SLE patients showed a higher peak of thrombin without and **B)** with 100 nM PMA stimulus and without CTI (an inhibitor of FXIIa). Presence of CTI decreased thrombin generation in control and SLE patients. p≤0.05 was stablished as statistical significance.

This work was supported by grants from the FIS-FONDOS FEDER (PI19/00772, NB). EMM holds a predoctoral fellowship from Federación Española de Trombosis y Hemostasia (FETH-SETH).

[Figure 2]

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UTILIDAD DE LAS TÉCNICAS GLOBALES DE LA COAGULACIÓN PARA DETECTAR EL PERFIL TROMBOGÉNICO DE PACIENTES CON LUPUS ERITEMATOSO SISTÉMICO SIN SÍNDROME ANTIFOSFOLÍPIDO

ha sido presentada como publicación por los autores

Monzón Manzano, Elena , Fernández Bello, Ihosvany, Justo Sanz, Raúl, Marhuenda, Ángel, Acuña Butta, Paula, Cebanu, Tamara, García Barcenilla, Sara, González Zorrilla, Elena, García Arias-Salgado, Elena, Álvarez Román, Mª Teresa, Jiménez Yuste, Víctor, Butta Coll, Nora

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331.PATHOPHYSIOLOGY OF THROMBOSIS | NOVEMBER 13, 2019

Prothrombotic State, Platelet Activation and Netosis in Systemic Lupus Erythematosus

Elena Monzón Manzano, Ihosvany Fernandez-Bello, Raul Justo Sanz, Larissa Valor, Francisco Javier López-Longo, Angel Robles, Teresa Álvarez Roman, Miguel A Canales, MD PhD, Victor Jimenez-Yuste, Nora Butta, PhD

Blood (2019) 134 (Supplement_1): 1141.
https://doi.org/10.1182/blood-2019-127991

Introduction: Systemic lupus erythematosus (SLE) is a chronic autoimmune disease of unknown origin characterized by a hypercoagulable state and a high mortality rate. Mechanisms that cause the accelerated deterioration of cardiovascular health in SLE are unknown.

Objectives: to characterize the prothrombotic state in SLE patients by global coagulation assays and the contribution of platelets, endothelial damage, microparticles and neutrophil extracellular traps (NETs) in their prothombotic profile.

Material and methods: 72 patients and 90 healthy controls were recruited. Patients were classified according to clinical characteristics in: 32 with lupus (SLE group), 29 with SLE and antiphospholipid antibodies (aFL, SLE+aFL group) and 12 who met the criteria for SLE and antiphospholipid syndrome (APS, SLE+APS group). Experimental protocol was approved by La Paz University Hospital Ethics Committee.

Venous blood collected in BD sodium citrate tubes (3.2%) was centrifuged at 150 g for 20 min at 23ºC to obtain platelet-rich plasma (PRP). PPP was obtained by centrifugation at 1500 g for 15 min at 23ºC.

To obtain neutrophils, whole blood was centrifuged to 1600 rpm 25 min using a Ficoll gradient and red cells were lysed.

Rotational thromboelastometry (ROTEM®) was performed in naTEM condition. Clotting time (CT, time from start of measurement until initiation of clotting [in seconds]); alpha angle (tangent to the curve at 2-mm amplitude [in degrees]), Ax (clot firmness at time x, [in mm]) and maximum clot firmness (MCF, [in mm]) were recorded.

Procoagulant activity associated to microparticle's content of tissue factor was determined in PPP by Calibrated Automated Thrombogram (CAT) using MP-reagent (4 mM phospholipids, Diagnostica Stago, Spain). We evaluated the endogenous thrombin potential (ETP, the total amount of thrombin generated over time); the lag time (the time to the beginning of the explosive burst of thrombin generation); the peak height of the curve (the maximum thrombin concentration produced) and the time to the peak.

Thrombin generation associated to NETs was also measured by CAT. Neutrophils from healthy controls or from LES patients were stimulated with 100 nM PMA in RPMI medium during 45 min at 37º and then cocultivated with PRP adjusted to 10^5 platelets/µL. NETs formation was verified by fluorescent microscopy performed with DAPI and an anti-myeloperoxidase antibody.

Plasma levels of LDL-ox, E-Selectin and PAI-1 were determined by Elisa (R&D Systems, MN, USA and Affymetrix eBioscience, Vienna, Austria, respectively).

Platelet activation was analysed by flow cytometry (FCM, FACScan, BD Biosciences). Fibrinogen receptor activation was evaluated through PAC1-FITC binding and release of granule's content was assessed with monoclonal antibodies (mAbs) anti-CD63 and anti P-selectin in quiescent and 100 µM TRAP and 10 µM ADP stimulated platelets.

Data were analysed with Graphpad prism and $p \leq 0.05$ was stablished as statistical significance.

Results: PAI-1 plasma level was increased in all patient's groups, whereas LDL-ox and E-selectin showed no differences with control cohort (Fig.1).

ROTEM demonstrated a procoagulant profile in SLE and SLE+aPL but not in SLE+APS group (Fig. 2). PAI-1 levels correlated with several ROTEM parameters (Table 1).

SLE patients and SLE+aFL showed a basal platelet activation. Moreover, SLE group exposed more P-selectin and CD63 than controls (Fig.3).

Regarding thrombin generation associated to tissue-factor content of microparticles, no differences were observed between SLE patients and healthy controls. On the other hand, SLE patients had an increased peak of thrombin generation related to NETs formation (control group: 170.3± 58.0, SLE patients: 230.6±39.3, p=0.019).

Conclusions: ROTEM® detected a hypercoagulable state in SLE and SLE+aPL patients. The hypercoagulable state might be linked to increased PAI-1 plasma levels and basal platelet activation in SLE and SLE+aPL groups. Moreover, neutrophils from SLE patients seemed to present a basal activation that induced a NETs-related procoagulant state in these patients.

SLE+APS patients did not show a hypercoagulable state perhaps because of the presence of lupus anticoagulant and/or to therapeutic treatment of these patients.

This work was supported by grants from the FIS-FONDOS FEDER (PI15/01457, NB). NVB holds a Miguel Servet tenure track grant from FIS-FONDOS FEDER (CP14/00024).

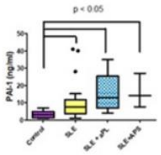

Figure 1. PAI-1 levels were increased in patients. Kruskal-Wallis test and Dunns post hoc test were applied and p≤0.05 was considered significant.

	A5	A20	A25	A30	MCF
PAI-1	r=0,36 p=0,05	r=0,352 p=0,048	r=0,411 p=0,024	r=0,344 p=0,05	r=0,418 p=0,019

Table 1. PAI-1 levels correlated with A5, A20, A25, A30 and MCF ROTEM® parameters. Analysis was performed using Spearman test. p ≤0.05 was denoted as significant.

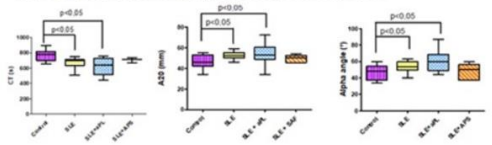

Figure 2. ROTEM® parameters showed a procoagulant profile in SLE and SLE+aPL groups. Kruskal-Wallis test and Dunns post hoc were applied considering p≤0.05 as significant.

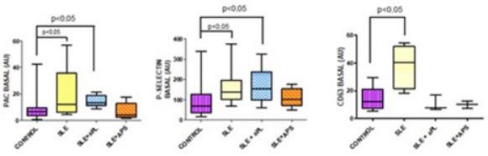

Figure 3. Activity of fibrinogen receptor and expression of P-selectin and CD63 on the platelet surface in basal condition. Data were expressed as arbitrary units (AU). Kruskal-Wallis test and Dunns post hoc were performed considering p≤0.05 as significant.

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Disclosures

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Topics: platelet activation, systemic lupus erythematosus, thrombin, plasminogen activator inhibitor 1, thrombophilia, antiphospholipid syndrome, cd63 antigen, e-selectin, loudness discomfort level, low-density lipoproteins

Author notes

* Asterisk with author names denotes non-ASH members.

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ESTADO PROTROMBÓTICO, ACTIVACIÓN PLAQUETARIA Y NETOSIS EN LUPUS ERITEMATOSO SISTÉMICO

ha sido presentada como comunicación por los autores

Monzón Manzano Elena, Justo Sanz Raúl, Fernández Bello Ihosvany, Acuña Butta Paula, García Barcenilla Sara, Cebanu Tamara, Valor Lara, Hernández Diana, López-Longo Francisco Javier, Jiménez Yuste Víctor, Butta Coll Nora

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ESTADO PROTROMBÓTICO Y ACTIVIDAD CLÍNICA EN PACIENTES CON LUPUS ERITEMATOSO SISTÉMICO

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CARACTERIZACIÓN DE LA HEMOSTASIA Y EL DAÑO VASCULAR EN LUPUS ERITEMATOSO SISTÉMICO MEDIANTE TÉCNICAS GLOBALES Y MARCADORES PLASMÁTICOS

ha sido presentada como **póster** por los autores

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9.3. Publicaciones

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Article

Insights into the Procoagulant Profile of Patients with Systemic Lupus Erythematosus without Antiphospholipid Antibodies

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Abstract: We aimed to identify the key players in the prothrombotic profile of patients with systemic lupus erythematosus (SLE) not mediated by antiphospholipid antibodies, as well as the potential utility of global coagulation tests to characterize hemostasis in these patients. Patients with SLE without antiphospholipid antibodies and without signs of thrombosis were included. The kinetics of clot formation were determined by ROTEM®. Platelet activation markers were determined by flow cytometry. Thrombin generation associated with Neutrophil Extracellular Traps (NETs) and microparticles (MPs) was measured by calibrated automated thrombogram (CAT). The plasma levels of PAI-1 were also determined. ROTEM® showed a procoagulant profile in SLE patients. SLE patients had activated platelets and more leukocyte/platelet aggregates at basal conditions. The plasma PAI-1 and platelet aggregates correlated with several ROTEM® parameters. The thrombin generation associated withthe tissue factor (TF) content of MPs and with NETs was increased. Our results suggest the utility of global tests for studying hemostasis in SLE patients because they detect their procoagulant profile, despite having had neither antiphospholipid antibodies nor any previous thrombotic event. A global appraisal of hemostasis should, if possible, be incorporated into clinical practice to detect the risk of a thrombotic event in patients with SLE and to consequently act to prevent its occurrence.

Keywords: systemic lupus erythematosus; thromboelastometry; thrombin generation; neutrophil extracellular traps

1. Introduction

Systemic lupus erythematosus (SLE) is a potentially fatal multiorgan inflammatory immune-mediated disease that primarily affects females. The disease is characterized by the production

of antibodies against various tissues, which triggers a wide variety of cutaneous and systemic manifestations that in many cases become serious, compromising the patient's life. Thrombosis contributes to substantial morbidity and mortality in patients with SLE due to a complex interplay between traditional risk factors and the dysregulation of autoimmunity. Up to 15% of patients with SLE have had myocardial infarction [1], and approximately 20–30% of deaths in patients with SLE are due to cardiovascular disease (CVD) [2,3]. Conflicting data on the mechanisms involved in the increase in risk and in the prediction of CVD complicate prevention of its occurrence.

The duration of the disease correlates with the degree of cardiovascular involvement [4], suggesting that chronic exposure to immune system dysregulation contributes to the development of CDV in these patients. The proposed predictors of cardiovascular events in this population are dyslipidemia, hypertension, a family medical history of coronary artery disease (CAD), and smoking. In addition, although the presence of antiphospholipid and anticardiolipin antibodies and lupus anticoagulant is correlated with the occurrence of cardiovascular events, 40% of SLE thrombosis cases are autoantibody-negative [5,6], suggesting the involvement of other factors.Thus, we aim to identify the key players in the prothrombotic profile of patients with SLE not mediated by antiphospholipid antibodies.

Recently, there has been growing interest in the use of global coagulation tests to evaluate hypercoagulable states [7,8]. Among them, rotational thromboelastometry (ROTEM®), a viscoelastometric clotting test that measures the kinetics of clot formation and fibrinolysis, and calibrated automated thrombogram (CAT), a thrombin generation test that quantifies thrombin generation, are the most widely used. Given that hemostasis is the consequence of the relationship between various cells, coagulation factors, and plasma components, we considered that these tests would be a good approach to evaluate the hypercoagulable condition in SLE. Therefore, we investigated the potential utility of ROTEM® and CAT in the characterization of the procoagulant state in SLE not mediated by antiphospholipid antibodies, giving a new insight into the relationship between different factors involved in this pathology.

2. Materials and Methods

2.1. Participants and Study Design

This study was approved by the ethics committees of two hospitals: Gregorio Marañón University Hospital (Code 324/14) and La Paz University Hospital (Code PI-3293). All the included patients had been diagnosed with SLE according to the American College of Rheumatology (ACR) criteria for SLE [9]. The global disease activity was measured with the Systemic Lupus Erythematosus Activity Index 2000 (SLEDAI-2K).

Exclusion criteria were infection with hepatitis C virus or human immunodeficiency virus; alcohol abuse or addiction; oral contraceptive intake or hormonal therapy (excepting steroids as an immunosuppressive treatment for SLE); patients with antiphospholipid antibodies (lupus anticoagulant, anti-β2-GPI, and anticardiolipin antibodies); a history of acute myocardial infarction, angina, or CAD; diabetes, hyperlipemia, or uncontrolled arterial hypertension; overweight defined by a body mass index ≥ 30 kg/m^2; smoking in the 12 months before our study; pregnancy in the previous 3 months prior to the study; or cancer.

Patients older than 18 years who fulfilled 4 or more ACR criteria, with a titer of antinuclear antibodies ≥1:80 and/or anti-double-stranded DNA antibody (anti-dsDNA antibody) levels ≥ 30 UI/mL, with a stable standard SLE therapy for the last 30 days, and who signed written informed consent were included in this study.

2.2. Collection and Preparation of Samples

Human peripheral blood samples were collected in tubes containing 3.2% trisodium citrate (BD Vacutainer, Madrid, Spain). Platelet-rich plasma (PRP) was prepared within 60 min of blood

collection by centrifugation (150 g for 20 min at 23 °C). To obtain platelet-poor plasma (PPP), the PRP was centrifuged twiceat 23 °C, first at 1500 g for 15 min and then at 13,000 g for 2 min.

Acid-citrate-dextrose (1:10) was added to the top two-third volumes of PRP and centrifuged at 650 g for 10 min at 23 °C to obtain washed platelets. The pellet was then resuspended in an equal volume of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (10 mM of HEPES, 145 mM of NaCl, 5 mM of KCl, and 1 mM of $MgSO_4$, pH 7.4).

For serum preparation, peripheral blood was collected in serum tubes (BD Vacutainer, Plymouth, UK) and separated by centrifuging clotted blood (2500 g for 15 min at 23 °C).

Plasma and serum aliquots were stored at −80 °C until analysis.

2.3. Cell Count and Biochemical Parameters

The blood cell count was performed using a Coulter AcT Diff cell counter (Beckman Coulter, Madrid, Spain). The plasminogen activator inhibitor type 1 (PAI-1) (Invitrogen, Vienna, Austria) levels were determined in serum or plasma according to the manufacturer's instructions and measured in a Multiskan FC microplate photometer (ThermoScientific, Madrid, Spain).

The cell-free DNA (cfDNA) was determined in PPP by the Quant-iT™ PicoGreen dsDNA assay (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

C-reactive protein (CRP), serum complement C3 and C4, erythrocyte sedimentation rate (ESR), creatinine, 24-hproteinuria, and anti-DNA titer were only determined in the SLE groups.

2.4. Rotational Thromboelastometry

The kinetics of clot formation and fibrinolysis were assessed by rotational thromboelastometry (ROTEM®, Pentapharm, Munich, Germany) with the recalcification of whole blood (NATEM®test).

The following parameters were recorded: clotting time (CT) (time from the start of clot formation until an amplitude of 2 mm, in seconds); alpha angle (α) (the slope of the tangent line to the clotting curve through the 20 mm amplitude that reflects the rate of fibrin polymerization, in degrees); the clot firmness X min after CT; the maximal clot firmness (MCF, in mm); and clot lysis as the percentage of clot lysedafter 60 min.

2.5. Analysis of Platelet Activation and Platelet Receptors

PRP was diluted 1:5 with HEPES buffer and incubated with either buffer, 100 μmol/L of thrombin receptor-activating peptide (TRAP)-6 (Bachem, Switzerland), or 10 μmol/L of adenosine diphosphate (ADP, Sigma, Madrid, Spain) at room temperature (RT). Later, fluorescein-isothiocyanate (FITC)-PAC1 (BD, Madrid, Spain), a monoclonal antibody (mAb) that recognizes only the activated conformation of fibrinogen receptor, FITC-anti P-selectin mAb (BD Pharmingen, San Diego, CA, USA), or FITC anti-CD63 mAb (BD, Madrid, Spain) were added and incubated for 15 min at RT. To determine the platelet receptors, diluted PRP was incubated with phycoerythrin (PE) anti-CD41 mAb (Biocytex, Marseille, France) or FITC anti-CD61mAb (BD, Madrid, Spain)—which recognized, respectively, the αIIb and β3 subunits of fibrinogen receptor—or it was incubated with FITC anti-CD42a mAb (BD, Madrid, Spain) or anti-CD42b mAb (BD Pharmingen, San Diego, CA, USA), against, respectively, the GPIX and GPIbα subunits of von Willebrand factor (vWF) receptor. These samples were analyzed using a FACScan flow cytometer (BD Biosciences, Madrid, Spain) after being diluted in 1:6 HEPES buffer.

2.6. Determination of Platelet-Leukocyte Aggregates

To determine the platelet-leukocyte aggregates, whole blood was diluted 1:10 in HEPES buffer and coincubated with FITC anti-CD45 mAb (BD Pharmingen, San Diego, CA, USA), PE anti-CD41 mAb, and 50 μM of TRAP or 40 μM of ADP for 15 min at RT in the dark. Platelet-leukocyte aggregates were defined as leukocytes positive for CD41.

2.7. Determination of Phosphatidylserine Exposure on Platelet Surface and Caspase Activity

The surface exposure of phosphatidylserine (PS) in washed platelets was assessed by measuring the binding of (FITC)-labeled Annexin V (BD Pharmingen, San Diego, CA, USA). Washed platelets were resuspended in annexinV binding buffer (10 mM of HEPES, 10 mM of NaOH, 140 mM of NaCl, 2.5 mM of $CaCl_2$, pH 7.4) and labeled with FITC-annexinV. After incubation for 15 min at RT in the dark, the samples were analyzed by flow cytometry.

To analyze the caspase-3, -7, -8, and -9 activity, PRP was diluted 10-fold with isotonic HEPES buffer containing 2 mM of $CaCl_2$ and 2 mM of Gly-Pro-Arg-Pro acetate (Sigma Aldrich, Madrid, Spain) to prevent fibrin formation, and either FAM-DEVD-FMK, FAM-LETD-FMK, or FAM-LEHD-FMK (Millipore, Madrid, Spain). The samples were analyzed by flow cytometry.

2.8. Calibrated Automated Thrombogram

The procoagulant activity of MPs associated with their content of either tissue factor (TF) or PS was determined, respectively, with MP reagent (4 μM of phospholipids) or PRP reagent (1 pM of recombinant human TF) by calibrated automated thrombogram (CAT). All CAT reagents were from Diagnostica Stago (Madrid, Spain). The thrombin generation was determined with a Fluoroskan FL instrument (ThermoLabsystems, Helsinki, Finland) under the control of Thrombinoscope software, version 3.6 (Thrombinoscope BV, Maastricht, Holand), filtered for excitation at 390 nm and emission at 460 nm.

The following parameters were determined: lagtime (LT) (time from the start of the assay until 10 nM of thrombin is formed, in min), time to peak (ttPeak) (time required to reach the maximum thrombin concentration, in min), peak height (Peak) (maximum thrombin concentration reached, in nM), and endogenous thrombin potential (ETP) (the total amount of thrombin generated over time, in nMxmin).

2.9. Neutrophil Isolation and Generation of Neutrophil Extracellular Traps

Neutrophils were isolated from 10 mL of whole blood from controls and from patients with SLE using a Percoll gradient centrifuged at 500 g for 25 min at 5 °C. The isolated neutrophils (2.5×10^6 cells/mL) were incubated with and without 100 nM of phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, Madrid, Spain) for 45 min at 37 °C in Roswell Park Memorial Institute (RPMI)-1640 medium (Invitrogen, Madrid, Spain). Later, the samples were centrifuged at 5000 g for 3 min and resuspended in PRP from healthy controls. The Neutrophil Extracellular Traps (NETs) formation was verified by fluorescence microscopy.

2.10. Assessment of Neutrophil Extracellular Trap Generation by Fluorescence Microscopy

Neutrophils were seeded on 12 mm cover glasses pretreated with poly-L-lysine (Sigma-Aldrich, Sweden) in 24-well plates in 500 μL of RPMI-1640 medium with and without 100 nM of PMA, for 45 min at 37 °C. The samples were fixed with a final concentration of 2% paraformaldehyde for 15 min at RT. Then, the preparations were blocked, adding 2% bovine serum albumin–phosphate-buffered saline for 45 min at RT and incubated first with a 1:300 dilution of rabbit anti-human myeloperoxidase (Dako, Madrid, Spain) and then with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Madrid, Spain) for 45 min at RT in dark. Finally, the samples were embedded in mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) and kept at 4 °C in the dark until visualization with fluorescence microscopy using the Nikon Eclipse 90i microscope.

2.11. Thrombin Generation Associated with Neutrophil Extracellular Traps

Blood from healthy controls was drawn in 2 tubes with citrate as an anticoagulant (Vacutainer, Madrid, Spain) and in 2 tubes with citrate plus 50 μg/μL of corn trypsin inhibitor (CTI) (Cell Systems Biotechnologie, Troisdorf, Germany) to inhibit activated factor XII (FXII). One of each kind of tube

was centrifuged to obtain PRP, and the others were centrifuged for PPP. PRP either with or without CTI was adjusted to 1×10^5 platelets/μL with the corresponding PPP. Neutrophils were isolated from healthy controls and from patients with SLE from citrated blood, as described above. Neutrophils were added to wells at a final concentration of 2.5×10^5 cells to 40 μL aliquots of PRP from healthy controls, with and without CTI, to perform CAT experiments after incubation for 30 min at 37 °C with either buffer or 100 nM of PMA without the addition of any trigger.

2.12. Statistical Analysis

The Shapiro–Wilk test was used to assess the distribution of the data, and the results were expressed as mean ± SD or median (p25–p75) depending on the distribution. The differences between the 2 groups were assessed using the 2-tailed unpaired Student's t-test or the non-parametric Mann–Whitney U-test, as appropriate. The correlation analysis was performed using Pearson's or Spearman's test. The GraphPad Prism 5 software (GraphPad Software version 5.03, GraphPad Software, San Diego, CA, USA) was used for all the statistical analyses, and significance was set at $p \leq 0.05$.

3. Results

3.1. Experimental Results

3.1.1. Features of the Patients with SLE

The study was performed on 32 patients with SLE treated at the Rheumatology Unit of the Gregorio Marañón University Hospital and at the Internal Medicine Unit of La Paz University Hospital, with a median age of 41.9 ± 12.9 years, who were recruited after signing informed consent. Eighty-eight sex- and age-matched healthy controls, with a mean age of 38.3 ± 11.6 years, were recruited as controls at the Blood Donation Center of La Paz University Hospital. The study was performed between January 2017 and October 2019. None of the patients had a history or signs or symptoms of thrombosis at inclusion.

A summary of the clinical and demographic data of the patients with SLE is shown in Table 1.

Table 1. Features of the patients with Systemic lupus erythematosus (SLE).

Patient	Disease Duration (Years)	Age (Years)	Medication at the Time of the Study	Concomitant Diseases	SLEDAI-2K
1	23	44	No treatment		12
2	21	50	Omeprazole, mycophenolate mofetil, prednisone, calcifediol	Autoimmune thrombocytopenia	6
3	9	35	Tramadol, levothyroxine, prednisone, rituximab	Sjogren's syndrome, Graves-Basedow disease, autoimmune hepatitis, fibromyalgia	3
4	18	35	Ramipril, phenelzine, abatacept, immunoglobulins		4
5	26	32	No treatment	Raynaud's phenomenon, endometriosis,	1
6	19	31	No treatment	Photosensitivity, inflammatory arthralgias, lymphopenia	5
7	13	49	Ferrous sulfate, omeprazole, prednisone, belimumab, azathioprine	Sjogren's syndrome	4

Table 1. *Cont.*

Patient	Disease Duration (Years)	Age (Years)	Medication at the Time of the Study	Concomitant Diseases	SLEDAI-2K
8	19	35	Quetiapine, duloxetine, omeprazole, diazepam, hydroxychloroquine, azathioprine, pregabalin, prednisone, tramadol, ferrous sulfate, calcifediol, rituximab		2
9	27	57	Prednisone, azathioprine, belimumab, rituximab		14
10	12	46	Calcifediol		2
11	4	45	Clobetasol propionate, trazodone, calcipotriol, diazepam, hydroxychloroquine, calcifediol, pregabalin, metamizole, omeprazole, almotriptan, enalapril, prednisone, sertraline, mycophenolate mofetil, abatacept, belimumab	Raynaud's syndrome, lupus nephropathy, mixed dyslipidemia	2
12	10	29	Azathioprine, hydroxychloroquine, prednisone, omeprazole, calcifediol, ferrous sulfate, belimumab	Sjogren's syndrome, leukopenia/lymphopenia	7
13	2	45	Hydroxychloroquine, prednisone, calcifediol, levothyroxine, azathioprine		0
14	15	33	Hydroxychloroquine, azathioprine, chondroitin sulfate		4
15	3	21	Hydroxychloroquine		4
16	23	61	Symbicort, enalapril, tramadol, pregabalin, azathioprine		0
17	10	31	No treatment		4
18	11	35	Methotrexate, omeprazole, folic acid, prednisone, mycophenolate mofetil		2
19	22	41	Nifedipine, hydroxychloroquine	Mixed connective tissue disease, Raynaud's syndrome	2
20	24	56	Abatacept, furosemide, fluoxetine, lorazepam, amisulpride, omeprazole, prednisone, spironolactone	Rheumatoid arthritis, Sjogren's syndrome, autoimmune hepatitis	4
21	6	67	Hydroxychloroquine, calcifediol		0

Table 1. *Cont.*

Patient	Disease Duration (Years)	Age (Years)	Medication at the Time of the Study	Concomitant Diseases	SLEDAI-2K
22	8	62	Hydroxychloroquine, omeprazole, levothyroxine, diazepam, paroxetine		2
23	23	58	Hydroxychloroquine, prednisone, atenolol, calcifediol		4
24	7	25	Hydroxychloroquine, calcifediol, Robaxisal		10
25	4	65	Hydroxychloroquine, prednisone, atorvastatin	Arterial hypertension	8
26	9	40	Mycophenolate mofetil, prednisone, hydroxychloroquine, enalapril, denosumab, ranitidine, paroxetine, calcifediol		0
27	8	22	Mycophenolate mofetil, ursodeoxycholic acid, hydroxychloroquine, calcifediol, ranitidine	Alpha-thalassemia minor, Raynaud's syndrome, secondary hyperhidrosis	4
28	22	48	Hydroxychloroquine, prednisone, calcifediol, omeprazole, cholecalciferol		2
29	12	40	Calcifediol		2
30	18	33	Prednisone, hydroxychloroquine, calcifediol	Atrial septal aneurysm, Kikuchi-Fujimoto disease, osteonecrosis	2
31	37	52	Omeprazole, hydroxychloroquine, amitriptyline, calcifediol	Depression	4
32	15	38	Hydroxychloroquine		0

Lymphocytes, erythrocytes, granulocytes, leukocytes, and platelet counts were reduced in the patients with SLE (Table 2). The CRP, C3 and C4 levels, ESR, creatinine, proteinuria (24-h), anti-DNA, IgA, IgM, and IgG levels were determined in most of the patients (Table 2).

3.1.2. Global Hemostasis in Patients with Systemic Lupus Erythematosus

To evaluate the global hemostasis and kinetics of clot formation, a ROTEM® test was performed using whole blood. Patients with SLE showed a procoagulant profile compared with the control samples (Figure 1). In the SLE patient group, we observed a shortening of the CT and a higher alpha angle, amplitude at 15 min, and MCF. No differences were found in the clot lysis at 60 min.

In order to determine whether MPs might participate in the procoagulant profile of patients with SLE, CAT was performed with different triggers that, according to the manufacturer, discriminate between thrombin generation dependent on the PS or TF content of MPs. As shown in Table 3, the ETP and peak of thrombin associated with the TF content of MPs was increased in patients with SLE. Moreover, the peak correlated to disease duration (Spearman r = 0.4634, $p = 0.0084$).

Table 2. Biochemical parameters in the healthy controls and patients with SLE.

	Controls	SLE	*p*-Value	Normal Range
Lymphocytes/μL	1.9 (1.6–2.4)	1.6 (1–1.8)	0.0093 *	1.2–3.4
Erythrocytes $\times 10^6$/μL	4.3 (4.1–4.6)	4.1 (3.8–4.4)	0.0421 *	4–6
Monocytes $\times 10^3$/μL	0.4 (0.3–0.5)	0.4 (0.2–0.4)	0.1046	0.1–0.6
Granulocytes $\times 10^3$/μL	4 (2.9–5.3)	2.9 (2.3–3.6)	0.0065 *	1.4–6.5
Leukocytes $\times 10^3$/μL	6.4 (5.3–7.6)	4.8 (4.2–5.7)	0.0012 *	4.5–10.5
Hemoglobin (g/dL)	13.2 (12.3–14.2)	12.9 (11.6–13.5)	0.1226	11–18
Platelets $\times 10^3$/μL	247 (208–284)	194 (171.5–231)	<0.0001 *	150–450
Hematocrit (%)	40.1 (38.4–43.7)	35.9 (34.4–39.8)	0.0004 *	35–60
MCV (fL)	94.6 (91.5–96.9)	92.4 (88.1–97)	0.2407	80–99.9
MCH (pg)	30.3 ± 1.6	28.6 ± 3.3	0.3037	27–31
MCHC (g/dL)	31.1 (31.3–32.9)	31.6 (30.7–32.3)	0.0976	33–37
RDW (%)	13.5 (12.8 –14.3)	14.1 (13.2–15.5)	0.1561	11.6–13.7
MPV (fL)	6.9 ± 0.8	7 ± 0.8	0.8931	7.8–11
Pct (%)	0.17 (0.15–0.19)	0.14 (0.12–0.19)	0.1210	0.190–0.36
PDW (%)	17.1 ± 0.8	17.3 ± 0.9	0.4936	0.190–0.36
CRP (mg/dL)	n.d	0.2650 (0.12–0.6)	-	0–0.5
C3 (mg/dL)	n.d	88.4 (71.5–106)	-	75–135
C4 (mg/dL)	n.d	16.3 (11.8–20.7)	-	14–60
Anti-DNA (mg/dL)	n.d.	14 (2.9–23)	-	<15.00
ESR (mm)	n.d	10.77 ± 0.6	-	2–20
Creatinine (mg/dL)	n.d	0.72 ± 0.12	-	0.5–0.9
IgG (mg/dL)	n.d.	1130 (910.5–1252)	-	725–1900
IgA (mg/dL)	n.d.	224.6 ± 92.32	-	50–350
IgM (mg/dL)	n.d.	84 (61.6–107.5)	-	45–280

Mann–Whitney or Student's t-tests were performed, and data are expressed as median (percentile 25%–percentile 75%) or mean ± SD depending on the sample distribution. A *p*-value ≤ 0.05 was set as significant, and * denotes significance. MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; RDW, red blood cell distribution width; MPV, mean platelet volume; Pct, plateletcrit; PDW, platelet distribution width; CRP, C-reactive protein; ESR, erythrocyte sedimentation rate; n.d, not determined.

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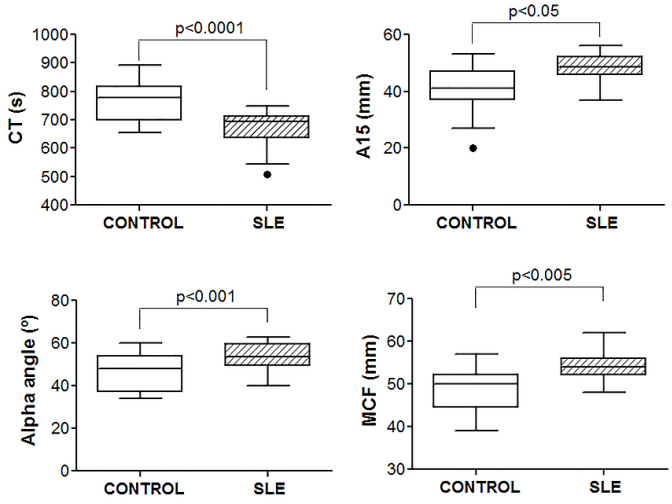

Figure 1. Procoagulant profile in patients with SLE. ROTEM® thromboelastography was performed in whole blood. Detailed procedures and measured parameters are shown in "Materials and Methods". Student's *t*-test or Mann–Whitney test was performed, and $p \leq 0.05$ was set as significant. SLE, systemic lupus erythematosus; CT, clotting time; A15, amplitude at 15 min; MCF, maximum clot firmness.

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Table 3. Microparticle-associated procoagulant capacity in patients with SLE.

	Controls	Patients with SLE	*p*
LT PRP-Reagent (min)	7.5 ± 3.8	7.8 ± 2.3	0.1280
Peak PRP-Reagent (nM)	90.5 ± 40.8	86.2 ± 52.5	0.1347
ttPeak PRP-Reagent (min)	12.1 ± 4.0	13.5 ± 3.2	0.1489
ETP PRP-Reagent (nM/min)	1021.0 ± 457.3	1107.0 ± 323.0	0.6076
LT MP-Reagent (min)	13.3 ± 3.2	13.2 ± 2.9	0.1186
Peak MP-Reagent (nM)	151.4 ± 36.5	253.8 ± 66.5	0.0021 *
ttPeak MP-Reagent (min)	16.1 ± 3.6	16.0 ± 3.3	0.1370
ETP MP-Reagent (nM/min)	1065.0 ± 241.8	1188.0 ± 313.6	0.0482 *

Data are expressed as mean ± SD. A p-value ≤ 0.05 was set as significant, and * denotes significance. Abbreviations: LT, lag time; ttPeak, time to peak; ETP, endogenous thrombin potential.

3.1.3. Platelet Activation in Patients with Systemic Lupus Erythematosus

Platelets have an essential role in clot formation; thus, we tested whether they were involved in the prothrombotic profile observed in the thromboelastogram of patients with SLE.

The platelets from patients with SLE presented basal activation, considering their increased PAC1 binding and major exposure of P-selectin and CD63 in quiescent conditions (Figure 2A). The basal activation of the platelets from patients with SLE was not the consequence of an increased expression of fibrinogen and vWF receptors on their surface (Figure S1). Moreover, we observed an increase in the platelet/leukocyte aggregate formation under basal conditions in the patients with SLE (Figure 2B). The percentage of platelet/leukocyte aggregate correlated with the ROTEM® parameters MCF (Spearman $r = 0.579$, $p = 0.030$) and alpha angle (Spearman $r = 0.532$, $p = 0.031$) and with the basal P-selectin exposure on the platelet surface (Spearman $r = 0.429$, $p = 0.035$), but did not correlate with the platelet and leukocyte counts. The platelet activation in patients with SLE did not depend on the platelet count. Moreover, the ROTEM parameters did not correlate with platelet activation markers.

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SLE (Figure 2B). The percentage of platelet/leukocyte aggregate correlated with the ROTEM® parameters MCF (Spearman r = 0.579, p = 0.030) and alpha angle (Spearman r = 0.532, p = 0.031) and with the basal P-selectin exposure on the platelet surface (Spearman r = 0.429, p = 0.035), but did not correlate with the platelet and leukocyte counts. The platelet activation in patients with SLE did not depend on the platelet count. Moreover, the ROTEM parameters did not correlate with platelet activation markers.

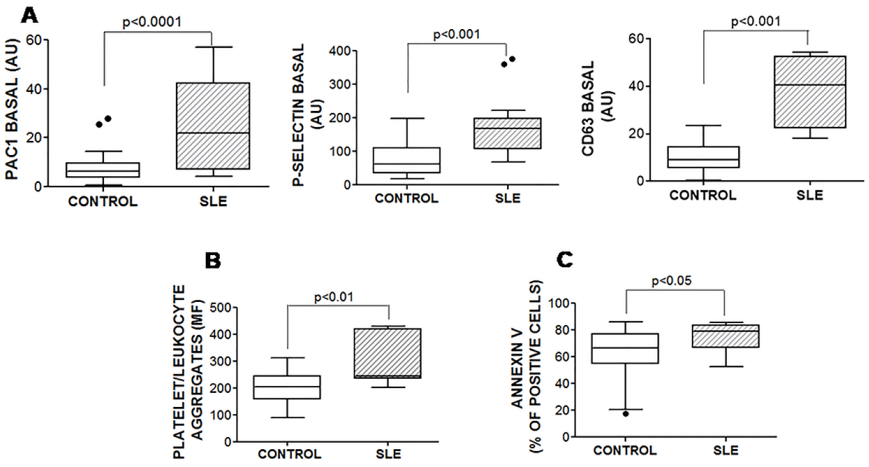

Figure 2. Basal activity of fibrinogen receptor, surface expression of P-selectin, platelet/leukocyte aggregate formation and PS exposure. (**A**) PRP from healthy controls and patients with SLE was incubated with either FITC-PAC1, FITC anti-P-selectin mAb, or FITC anti-CD63 mAb. (**B**) To test the platelet/leukocyte aggregates, whole blood was incubated with PE anti-CD41 mAb and FITC anti-CD45 mAb. (**C**) Annexin V binding was tested in washed platelets resuspended in the adequate buffer (see Methods). Samples were analyzed by flowcytometry. The Mann–Whitney test was performed, and $p \leq 0.05$ was considered significant. Data are expressed as arbitrary units (mean fluorescence ×% of positive cells (**A**), mean fluorescence of leukocytes positive for CD41 (**B**), or percentage of positive cells (**C**)).

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The response to activation with either 100 μM of TRAP or 20 μM of ADP was similar in the platelets from healthy controls and from patients with SLE (Figure S2).

3.1.4. Phosphatidylserine Exposure and Apoptosis in SLE Platelets

Platelets from patients with SLE bound more annexin on their surface than the controls, indicating an enhanced PS exposure (Figure 2C). This fact did not appear to be related to enhanced apoptosis, because the caspase activities were similar among groups (Figure S3).

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3.1.5. Association between Coagulation Profile and Inflammatory State

An association between coagulation and inflammatory states has been reported [10]. Therefore, we tested the plasma levels of PAI-1, which is considered a marker of vascular inflammation [11]. The PAI-1 levels were increased in patients with SLE (Figure 3A).

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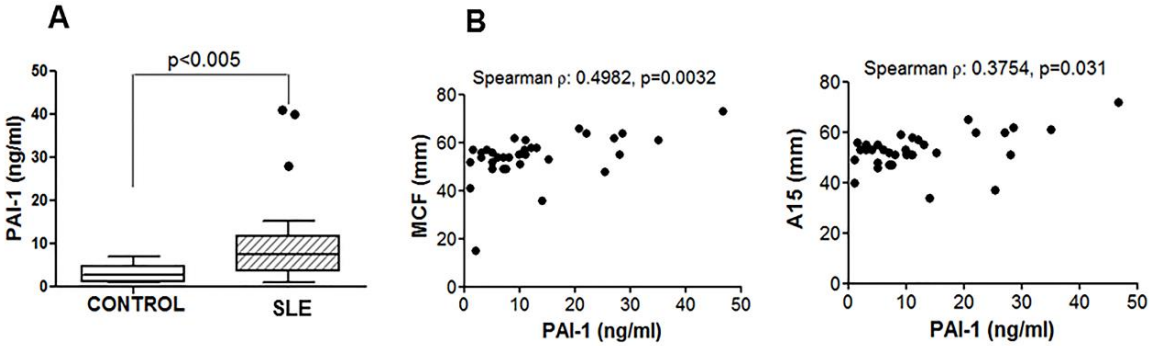

Figure 3. Plasminogen activator inhibitor-1 (PAI-1) levels in plasma and its correlation with ROTEM® parameters. Plasma levels of PAI-1 (**A**) measured with enzyme-linked immunosorbent assay correlated with A15 and MCF parameters (**B**). A Mann–Whitney test and Spearman's correlation were performed, and $p < 0.05$ was considered significant.

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Furthermore, the PAI-1 levels correlated with the ROTEM® parameters A15 and MCF (Figure 3B), suggesting an association between the PAI-1 plasma levels and the procoagulant state observed in patients with SLE. On the contrary, the PAI-1 levels did not correlate with the disease activity index.

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3.1.6. Thrombin Generation Associated with Neutrophil Extracellular Trap Formation

The plasma content of nucleic acids might contribute to the creation of prothrombotic profiles. We observed that the plasma from patients with SLE had increased cfDNA in fluorescence units, controls: 94.90 ± 21.29, SLE patients: 112.4 ± 26.59; $p = 0.0211$). In accordance with this observation, the neutrophils from SLE patients, but not the controls, showed NETs in basal conditions (Figure 4). Moreover, the neutrophils from these patients generated more NETs in the presence of 100 nM of PMA, as confirmed by fluorescence microscopy (Figure 4).

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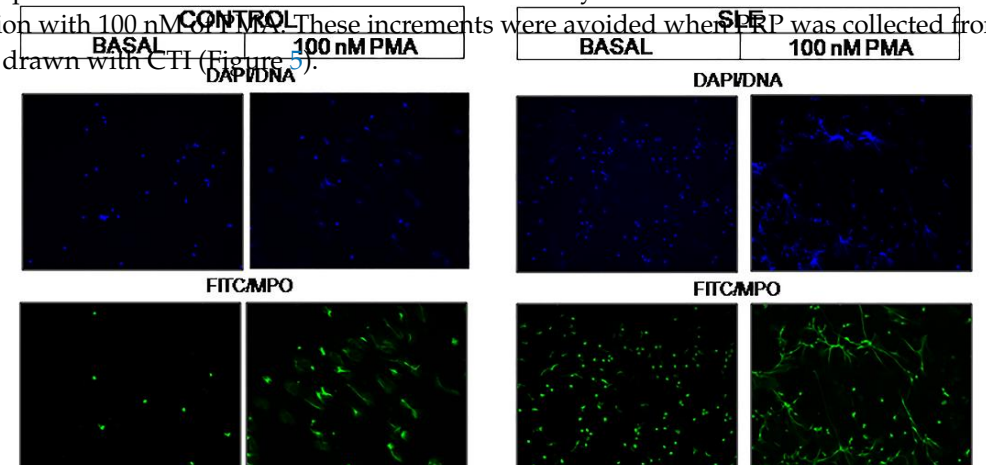
To evaluate whether the increment in NETs observed in patients with SLE had consequences on the hemostasis of these patients, we tested the thrombin generation of neutrophils from either patients with SLE or controls in the presence of platelets from healthy controls. The neutrophils from patients with SLE produced more thrombin than those from healthy controls under basal conditions and after stimulation with 100 nM of PMA. These increments were avoided when PRP was collected from blood samples drawn with CTI (Figure 5).



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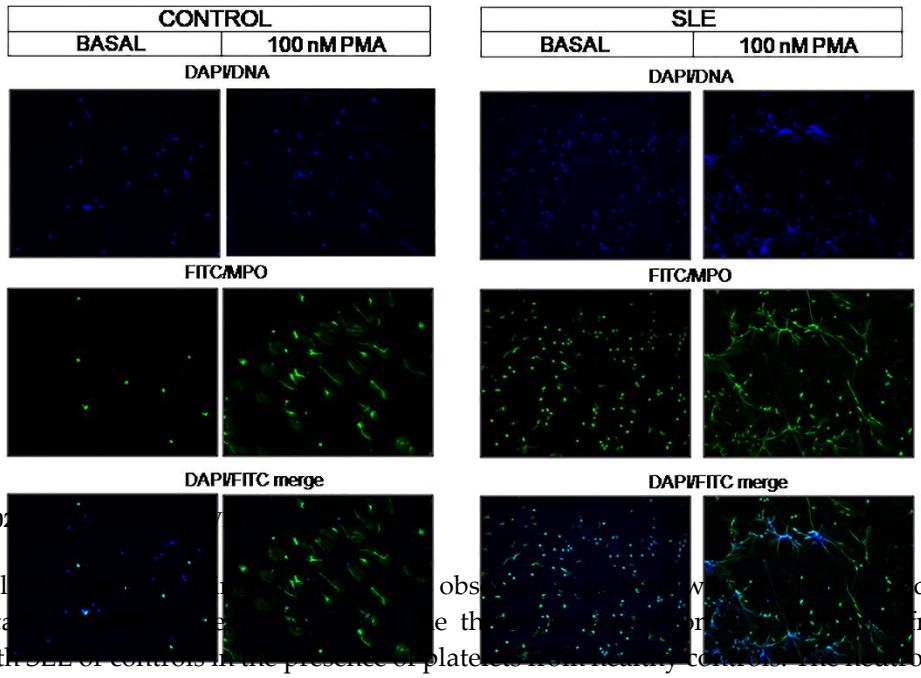

Figure 4. Formation of NETs NETs were evaluated in basal conditions and after stimulation with 100 nMol PMA. DNA (DAPI, blue), neutrophil myeloperoxidase (MPO, FITC, green), and DAPI/FITC merge images areshown. Original magnification ×10.

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To evaluate the influence of NETs on the hemostatic profile, we observed the effects on the thrombin generation from either patients with SLE or controls in the presence of platelets from healthy controls. The neutrophils from patients with SLE produced more thrombin than those from healthy controls under basal conditions and after stimulation with 100 nM of PMA. These increments were avoided when PRP was collected from blood samples drawn with 0.1 U/mL of DNase I (Figure 5).

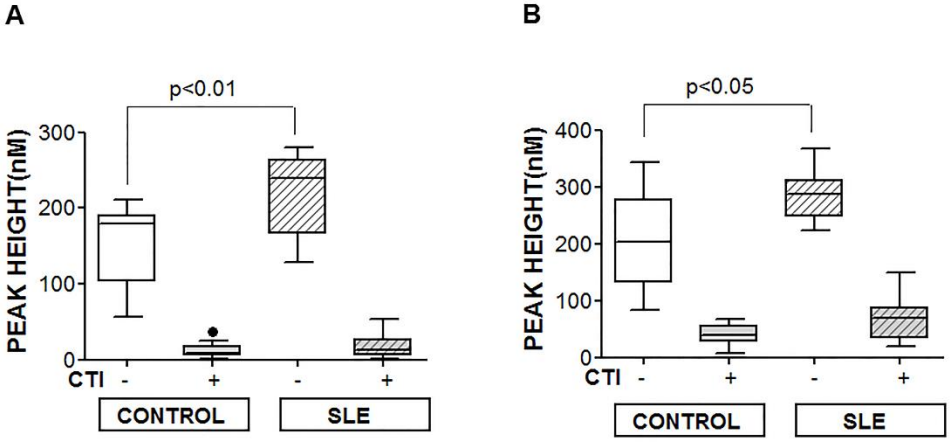

Figure 5. Thrombin generation associated with NETs. The effect of NETs on the thrombin generation was tested in either non-stimulated (**A**) or 100 nM of PMA-stimulated neutrophils (**B**) in the presence of PRP from healthy controls adjusted to 1×10^5 platelets/µL, with (+) or without (−) CTI. Detailed procedures are explained in "Materials and Methods". A Mann–Whitney test was performed, and $p < 0.05$ was considered significant.

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4. Discussion

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Analyses of the ROTEM® parameters in our cohort of patients showed a shortened CT and an increased alpha angle and MCF, highlighting the hypercoagulable features of these patients despite the fact that they had no antiphospholipid antibodies and no history of suffering thrombotic events. However, K.S. Collins et al. [12], who tested the kinetics of clot formation employing another global method, thromboelastography (TEG), found no differences in the TEG parameters between patients with SLE and healthy controls. This discrepancy could be due to the trigger used to induce coagulation in each case. These authors employed kaolin to activate the clotting cascade. Kaolin is a more powerful trigger, but offers a minor sensitivity in detecting mild differences in coagulation kinetics. Differently, we used the non-activated rotational thromboelastometry, NATEM®, which is sensitive to any change in the balance of the coagulation system, but has a low specificity [13]. Interestingly, other authors have evaluated coagulation by TEG in a cohort of children with SLE, and they also found a procoagulant profile; in this study, however, patients with antiphospholipid

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In some of the patients from our cohort, SLE was accompanied by other syndromes such as Raynaud and Sjögren, known to induce a procoagulant state [15,16] that might overlap with that produced by SLE. Nevertheless, this does not seem to occur, because no differences were found among groups of SLE patients without and with these accompanying syndromes.

We did not find a correlation between the activity index of the disease and the patients' procoagulant profile. A similar conclusion was drawn from a systematic review and meta-analysis performed by Balloca et al. [17]. Despite the lack of correlation between the disease activity and procoagulant profile, we compared the clinical features of SLE patients with an MCF higher than the mean value + SD of MCF from healthy controls with those with an MCF within the normal range. We observed that, while both groups had similar nephrological damage, those with a high MCF had approximately 1.5 times more muscular and cardiac compromise and three times more pulmonary and nervous system clinical manifestations. Even when these data should be verified increasing the number of patients in the cohort, our observation warns about the importance of maintaining patients with hemostatic and coagulation parameters within normal ranges. Accordingly, some authors have proposed to treat patients with SLE with prophylactic oral anticoagulant therapy [18].

Platelet number and function are important determinants in the kinetics of clot formation [12,19]. The platelet counts in our SLE cohort, despite being significantly lower than in the healthy control group, were within a normal range. Thus, this difference was not expected to alter the ROTEM® parameters and, if any effect was predictable, it was a hypocoagulable one.

Treatment in SLE aims at remission or low disease activity and the prevention of flares. Our cohort of patients with SLE was treated according to the EULAR recommendations [20]. In patients without treatment, disease was in remission. Nevertheless, due to the complex and diverse nature of SLE pathogenesis, most of the patients need a combinatory therapy that may include standard immunosuppressive drugs (corticosteroids, azathioprine, methotrexate, cyclophosphamide, and mycophenolate) and monoclonal antibodies blocking CD20 (rituximab) or B-cell-activating factors (belimumab). In particular, one of our patients received at the same time rituximab and belimumab. This combination has been recently tested and demonstrated to reduce immune-complex-mediated inflammation and NETs formation [21].

Most of the patients with SLE from our cohort were treated with hydroxychloroquine, a substance known to have inhibitory effects on platelets [22]. Nevertheless, we and other authors [23] have observed that platelets from patients with SLE are basally activated. This activation could be due, at least in part, to the effect of the anti-dsDNA antibodies present in patients with SLE that may induce platelet activation, demonstrated by the enhanced P-selectin expression and morphological platelet changes [24]. However, the platelets from patients with SLE showed a reduced exposure of P-selectin after TRAP and ADP stimulation. Similarly, Frelinger et al. had reported that platelets from children with immune thrombocytopenia showed an increased P-selectin exposure in quiescent conditions but not after stimulation with agonists [25]. This observation might be because the basal activation of platelets causes either a reduction in the number or the exhaustion of secretory α-granules.

Another consequence of the basal activation of platelets from patients with SLE is the enhancement of their interaction with leukocytes (present results and other authors [26,27]) through the binding of P-selectin with the PSGL-1 present on the leukocytes' surface. Once bound, the interaction between the integrin αMβ2 on leukocytes and the GPIb on platelets produces a firm adhesion that contributes to thrombosis [28]. Moreover, platelet–leukocyte interactions induce signals that amplify proinflammatory cellular responses [29,30].

In accordance with their basal activation [31], platelets from patients with SLE exposed more PS than platelets from healthy controls (Figure 2C). Given that the PS exposition on the surface of

platelets provides a negatively charged scaffold for the binding of the tenase and/or prothrombinase complexes that promote thrombin generation [32], it is tempting to speculate that this is an additional mechanism through which platelets could participate in the procoagulant profile of patients with SLE. The PS exposure on platelets from patients with SLE was due to activation and not to apoptosis [33], because the caspase activities in their quiescent platelets were similar to those observed in platelets from the healthy controls.

The procoagulant profile in patients with SLE might also be due to the presence of MPs released by cells in response to activation or apoptosis. Given that SLE is characterized by chronic inflammation and tissue damage, it is not surprising that blood from patients with SLE contains more MPs [34–36], due to both activated and damaged cells. MPs support coagulation by the exposure of negatively charged phospholipids and TF. We observed that our cohort of patients with SLE generated more thrombin associated with the TF content of MPs, whereas no differences were observed in thrombin generation linked to the PS content of MPs. These results might be explained because MPs from patients with SLE are predominantly PS-negative [37]. Nevertheless, Pereira et al. has reported an increased thrombin generation dependent on PS-associated MPs [36]. Moreover, other authors have described the augmentation of MPs in patients with SLE, measuring their PS exposure [38]. These differences might rely on the inclusion criteria for patients with SLE or/and the use of distinct technical approaches for measuring MPs. Given that the MPs in SLE exhibit unique molecular and phenotypic features, including the infrequent expression of PS, their role in the pathogenesis of SLE and their utility as biomarkers have been suggested by many authors [35,39].

On the basis of the existence of a correlation between the procoagulant profile (the peak of thrombin generation associated with MPs' TFcontent) and disease duration (but not with the disease activity index), it is tempting to speculate that chronic damage is more important than the current disease activity for the hypercoagulable state of these patients. Nevertheless, it cannot be ruled out that lack of correlation between coagulation parameters and disease activity might be due to our small sample size.

The increase in TF-bearing MPs could come from damaged endothelial cells [40]. In support of this observation, we and other authors [35,39] have observed increased plasma levels of PAI-1, a marker of endothelial dysfunction, in patients with SLE. In addition, augmented PAI-1 accompanied higher MCF values.

The damage to endothelial cells in SLE could be due, in part, to the presence of elevated levels of a pathogenic neutrophil subset known as low-density granulocytes, which have been reported to contribute to lupus pathogenesis through heightened proinflammatory responses, altered phagocytic capacity, and vascular damage [41]. Moreover, this neutrophil subset tends to release more web-like NETs [42,43], which are composed of cfDNA, histones, antimicrobial proteins, fibrinogen, FXII, and TF [43,44]. This release explains the increased levels of cfDNA found in plasma from patients with SLE. Moreover, most of the patients with SLE have a reduced ability to degrade NETs [42], and the prolonged presence of NETs in plasma could promote a rupture of immune tolerance as well as increase tissue damage [45]. These events lead to the formation of an amplification loop, in which NET components induce autoantibodies, leading to the formation of more immune complexes which, in turn, perpetuate NET formation. The experiments of Etulain et al. performed on mice showed an increase in NETs in the presence of P-selectin and PSGL1 [46]. This could explain why the neutrophils from patients with SLE were more susceptible to generating NETs due to basal platelet activation.

Moreover, enhanced NET formation has been associated with the promotion of coronary plaque formation and lipoprotein dysregulation [47].

The increased thrombin generated by non-stimulated neutrophils from patients with SLE (present results) could be explained by the fact that cfDNA triggers the intrinsic pathway of blood coagulation [48]. NETs can bind FXII and cooperate with platelets to activate the intrinsic pathway [49]. In support of this observation, NET-related thrombin generation was prevented in the presence of CTI, an inhibitor of the contact phase of coagulation activation (present results and [50]).

One of the limitations of the study is that the patients were receiving different treatments that might modify hemostasis, as mentioned above for hydroxychloroquine, and that we did not recruit enough patients for stratifying them according to the medication they were receiving.

Traditional cardiovascular risk factors do not fully explain the high rates of ischemic events in patients with SLE, and standard risk calculations underestimate the risk of developing cardiovascular disease. Previous reports have already shown similar results to those presented in this work—platelets from SLE patients are activated [27] and increments were observed in circulating MPs [36], platelets/leukocyte aggregates [26], and PAI-1 [39] and cfDNA [51] plasma levels. Importance of our work relies on the fact that all these variables were evaluated in the same cohort of patients, allowing us to detect the relationship between the different mechanisms involved. In addition, the effects observed were independent of antiphospholipid antibodies because their presence was an exclusion criterion. Another key point is that our results suggest the utility of global tests for studying hemostasis in these patients, because a procoagulant profile was detected despite the fact that they had neither antiphospholipid antibodies nor any previous thrombotic event. A global appraisal of hemostasis takes into account the relationships among all the mechanisms involved (platelets, the thrombin generation associated with MPs, cfDNA) and should, if possible, be incorporated into clinical practice to detect the risk of a thrombotic event in patients with SLE and to consequently act to prevent its occurrence, as recommended in the updated guide of EULAR [20].

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-0383/9/10/3297/s1, Figure S1: Fibrinogen and von Willebrand factor (vW Factor) receptors expression on quiescent platelets. Platelets were incubated with PE-anti CD41 and FITC-anti CD61 mAbs to detect fibrinogen receptor and with FITC-anti CD42a and FITC-anti CD42b mAbs to test vW Factor receptors. Data are expressed as mean fluorescence (MF). Samples were analyzed by flow cytometry. Figure S2: Platelet's activation markers. Platelets were stimulated with either 100 μM of TRAP or 20 μM of ADP, and FITC-PAC1, FITC-anti P-selectin mAb, and FITC-anti CD63 mAb were added. Data are expressed as % of positive cells. Samples were analyzed by flow cytometry. Figure S3: Caspase activities in quiescent platelets. Data are expressed as % of positive cells. Samples were analyzed by flow cytometry.

Author Contributions: I.F.-B., E.M.M., R.J.S., and P.A.; F.J.L.-L. and Á.R.M. recruited patients and collected clinical data; I.F.-B., M.T.Á.R., V.J.Y., Á.R.M., and N.V.B. analyzed data of work, N.V.B. designed the study and wrote the manuscript. All the authors revised critically the paper and approved it. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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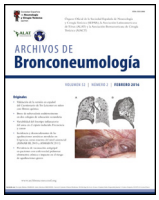

Original Article

Procoagulant State of Sleep Apnea Depends on Systemic Inflammation and Endothelial Damage

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ABSTRACT

Introduction: Growing evidence shows a hypercoagulable state in obstructive sleep apnea (OSA) that could be a risk factor for thromboembolic disease.
Objectives: We aimed to elucidate mechanisms involved in the procoagulant profile observed in patients with OSA and to investigate the potential utility of global tests in its characterization.
Methods: Thirty-eight patients with severe OSA without previous history of thrombosis and nineteen healthy age- and sex-matched controls were included.
Kinetic of clot formation was determined using rotational thromboelastometry.

Haemostatic capacity of plasma and microparticles was determined by Calibrated Automated Thrombinography.

Platelet surface receptors, activation markers and formation of platelet/leukocytes aggregates were analyzed by flow cytometry.
Results: Thromboelastometry showed a procoagulant state in patients with OSA that did not seem to be related to a basal activation of platelets but by the increased existence of platelet/leukocyte aggregates. Patients with OSA presented many signs of endothelial damage such as increased plasma levels of E-selectin and cfDNA and enhanced thrombin generation due to the presence of microparticles rich in tissue-factor, which is related to OSA severity.
Conclusions: OSA induces an enhancement in the dynamics of clot formation which appears to be caused by at least two pathological mechanisms. First, a greater formation of platelet-leukocyte aggregates; secondly, endothelial damage which provokes a greater procoagulant potential due to the increase in tissue factor-rich microparticles. Moreover, this study has identified thromboelastometry and thrombin generation assay as useful tools to evaluate the prothrombotic state in these patients.

El estado procoagulante de la apnea del sueño depende de la inflamación sistémica y el daño endotelial

Palabras clave:
Apnea obstructiva del sueño
Generación de trombina
Tromboelastometría

RESUMEN

Introducción: Existen pruebas crecientes que muestran un estado de hipercoagulabilidad en la apnea obstructiva del sueño (AOS) que podría ser un factor de riesgo de desarrollar enfermedad tromboembólica.

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Plaquetas
Micropartículas ricas en factor tisular

Objetivos: Nuestro objetivo fue dilucidar los mecanismos involucrados en el perfil procoagulante que se ha observado en los pacientes con AOS e investigar la posible utilidad de las pruebas globales en su caracterización.
Métodos: Se incluyeron 38 pacientes con AOS grave sin antecedentes de trombosis y 19 controles sanos emparejados por edad y sexo.
La cinética de la formación del coágulo se determinó mediante tromboelastometría rotacional.
La capacidad hemostática del plasma y las micropartículas se determinó mediante trombinografía automatizada calibrada.
Los receptores de la membrana plaquetaria, los marcadores de activación plaquetaria y la formación de agregados de plaquetas-leucocitos se analizaron mediante citometría de flujo.
Resultados: La tromboelastometría mostró un estado procoagulante en pacientes con AOS que no parecía estar relacionado con una activación basal de las plaquetas, sino por el aumento de agregados de plaquetas-leucocitos.
Los pacientes con AOS presentaban muchos signos de daño endotelial, como un aumento de los niveles plasmáticos de E-selectina y ADNcf y una mayor generación de trombina debido a la presencia de micropartículas ricas en factor tisular, que se relaciona con la gravedad de la AOS.
Conclusiones: La AOS induce un aumento de la dinámica de la formación de coágulos que parece estar causada por al menos 2 mecanismos patológicos. Primero, una mayor formación de agregados plaquetas-leucocitos; segundo, el daño endotelial que provoca un mayor potencial procoagulante debido al aumento de micropartículas ricas en factor tisular. Además, este estudio ha identificado la tromboelastometría y el ensayo de generación de trombina como herramientas útiles para evaluar el estado protrombótico en estos pacientes.

Introduction

Obstructive sleep apnea (OSA) is a highly prevalent disorder characterized by repetitive episodes of partial or complete upper airway closure during sleep leading to intermittent hypoxia and sleep fragmentation.[1] These immediate alterations trigger a cascade of mechanisms, such as increased sympathetic activity, altered vascular regulation, endothelial dysfunction, oxidative stress, and chronic systemic inflammation, which increase the risk of cardiovascular disturbances.[2] In fact, OSA is associated with a high incidence of arterial hypertension, stroke, ischemic heart disease, arrhythmias, and heart failure.[3]

There is growing evidence that OSA could also be a risk factor for venous thromboembolic disease (VTD).[4] This association represents a major public health burden due to its high prevalence and mortality. The overall annual incidence rate of VTD reaches 75–269 cases per 100,000 persons, and its risk approximately doubles with each decade after the age of 40 years.[5] Moreover, pulmonary embolism, the primary manifestation of VTD, is the third most frequent cardiovascular disease.[5]

Although some risk factors for OSA, including obesity, increasing age, and sedentary lifestyle are the same as those for VTD [5]there is increasing evidence of a hypercoagulable state in OSA.[6,7] Some studies have suggested significant associations between OSA and increased fibrinogen and D-dimer[6] and increased concentrations of coagulation factors (including thrombin and antithrombin, and von Willebrand factor),[8] higher serum levels of tissue factor [8]increased platelet activity and aggregability,[9,10] impaired fibrinolytic activity [7]and whole-blood hypercoagulability. However, hemostasis is a complex process that encompasses blood clotting, platelet activation, and vascular repair, involving multiple molecules and cell types, such as platelets, endothelium cells, and leukocytes; thus, its evaluation requires an integrated examination. Here, we investigated the potential utility of rotational thromboelastometry (ROTEM) and calibrated automated thrombogram (CAT) in the characterization of the procoagulant state in patients with severe OSA and assessed whether these tests offer new insight into the physiopathological mechanisms of OSA and its procoagulant profile.

Methods

Newly diagnosed OSA patients over 35 years of age with an apnea-hypopnea index (AHI) $\geq$ 30/h on respiratory polygraphy and without previous comorbidities or history of thrombosis were consecutively included. Healthy volunteers without OSA, who were homogeneous in sex, age and smoking habit were selected as controls.

The study was approved by the local Ethics Committee (registry number PI-1857), and informed consent was obtained from all participants. A more detailed description of the methods is provided in the online data supplement.

Platelet-free plasma (PFP) and platelet-rich plasma (PRP) were prepared as explained in the online data supplement.

Rotational thromboelastometry was performed in whole blood by ROTEM Gamma (Pentapharm, Munich, Germany). ROTEM evaluates clotting time (CT: time from start of test until 2 mm of amplitude, in seconds); clot formation time (CFT: time from CT to an amplitude of 20 mm, in seconds); α angle (tangent to the curve at 20 mm amplitude, in degrees); amplitude at "$\times$" time (in mm); maximum clot firmness (MCF: reflects the maximum strength of the clot, in mm); maximum velocity of clot formation (MAXV), and lysis at 60 min (LI60, indicates residual clot firmness at 60 min, in %).

Surface expression of fibrinogen and von Willebrand factor receptors as well as activation markers (activation of fibrinogen receptor and P-selectin and tetraspanin CD63 release from alpha and dense granules, respectively), and basal and thrombin receptor-activating peptide 6 (TRAP)- and adenosine diphosphate (ADP)-induced leukocyte-platelet aggregates were determined by flow cytometry, as described in online data supplement.

Thrombin generation was assessed in platelet-free plasma (PFP) in fresh by calibrated automated thrombogram (CAT) employing PPP-Reagent LOW. Procoagulant activity associated with microparticle (MP) content of either tissue factor (TF) or phosphatidylserine (PS) was determined using, respectively, MP-reagent and PRP-reagent. The lagtime (time from start of test until 10 nM thrombin was formed, in min), the peak height of the curve (the maximum thrombin concentration generated, in nM); the time to reach the peak and endogenous thrombin potential (total amount of

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Table 1
Baseline characteristics of the study participants.[b]

	Severe OSA group ($n = 38$)	Healthy volunteers ($n = 19$)	p-Value[a]
Age, years	60 ± 11	58 ± 11	0.427
Males, n (%)	28 (74)	12 (63)	0.301
Body mass index, kg/m²	31.9 ± 5.2	28.6 ± 3.2	0.013
Neck circumference, cm	38 (33–43)	36 (32–38)	0.038
Smoking habit, n (%)			0.928
Current smoker	11 (29)	5 (26)	
Former smoker	13 (34)	6 (32)	
Never smoker	14 (37)	8 (42)	
Epworth Somnolence Score (ESS)	8 ± 5	3 ± 2	<0.001
Daytime sleepiness (ESS ≥ 10)	13 (34)	0	<0.001
AHI, events/h	48.5 ± 16.5	2.8 ± 1.2	<0.001
Oxygen desaturation index, events/h	47.0 ± 17.6	2.9 ± 0.9	<0.001
$tSpO_2$ < 90%, %	28.4 ± 24.5	0.5 ± 0.8	<0.001
Mean nocturnal SpO_2, %	91 ± 2	95 ± 1	<0.001
Low nocturnal SpO_2, %	75 ± 9	89 ± 1	<0.001
Obstructive events, %	87.2 ± 6.4	86.0 ± 6.4	0.343
Systolic blood pressure, mm Hg	124 ± 10	122 ± 10	0.359
Diastolic blood pressure, mmHg	85 ± 7	73 ± 6	0.421
Red blood cell count × 10^6, per μl	4.5 ± 0.5	4.4 ± 0.5	0.560
Hemoglobin, g/dl	14.1 ± 1.3	13.4 ± 1.7	0.173
Hct, %	42.9 ± 4.9	42.1 ± 3.5	0.339
MCH, pg	31.2 ± 1.3	30.3 ± 1.8	<0.05
MCHC, g/dl	33.0 ± 0.6	31.2 ± 1.3	<0.0001
RDW, %	13.2 ± 0.6	15.0 ± 1.8	<0.0001
White cell count × 10^3, per mm^3			
Total	6.5 ± 1.7	6.7 ± 1.7	0.333
Neutrophils	4.0 ± 1.3	4.4 ± 1.3	0.204
Lymphocytes	2.1 ± 0.6	2.0 (1.8–2.2)	0.828
Monocytes	0.3 (0.2–0.5)	0.5 ± 0.5	0.156
Platelet count × 10^3, per μl	213.6 ± 64.3	216 (180–238)	0.847
MPV, fl	7.1 ± 0.5	7.4 ± 0.7	0.116
PDW, %	17.4 ± 0.8	17.3 ± 0.9	0.757
Cholesterol total, mg/dl	186 ± 50	173 ± 33	0.320
HDL-cholesterol, mg/dl	124 ± 46	113 ± 30	0.352
LDL-cholesterol, mg/dl	51 ± 6	50 ± 4	0.855
Triglycerides, mg/dl	151 ± 49	129 ± 20	0.065

Abbreviations: AHI = apnea–hypopnea index; ESS = Epworth somnolence score; HDL = high-density lipoprotein; LDL = low-density lipoprotein; Hct = hematocrit; MCH = mean corpuscular hemoglobin; MCHC = mean corpuscular hemoglobin concentration; MPV = mean platelet volume; PDW = platelet distribution width; RDW = red cell distribution width; SpO_2 = oxyhemoglobin saturation; $tSpO_2$ < 90% = time with SpO_2 < 90%.

[a] Comparisons between groups were performed by Student's *t*, Mann–Whitney *U*, or chi-squared test.

[b] Values are mean ± SD, median (interquartile range) or percentage, according to their distribution.

thrombin generated over time, in nMxmin) were determined. More details are given in the in online data supplement.

E-selectin and cell free DNA (cfDNA) were quantified, respectively, by ELISA (R&D Systems Europe Ltd., Abingdon, UK) and by a fluorometric test in PFP as mentioned in online data supplement.

Data were analyzed by GraphPad Prism software 5.03. Normal distribution of the data was tested using the Shapiro–Wilk test. Mann–Whitney or Student's *t*-tests were performed, and data were expressed as median (percentile 25%–percentile 75%) or mean ± standard deviation depending on sample distribution.

The correlation analysis was performed using Pearson's or Spearman's test. All tests were two-tailed, and statistical significance was set at $p < 0.05$.

Comparisons between OSA patients and control subjects were adjusted by anthropometric characteristics by linear general model univariate analysis, using group as fixed factor and sex, age, BMI and neck circumference as covariates.

Results

Patient population

Thirty-eight patients with severe OSA and nineteen healthy age- and sex-matched controls were included. The clinical characteristics of the patients are summarized in Table 1. Although patients with OSA had a higher BMI than control subjects, the percentage of obese patients was similar in both groups (51.4 vs. 42.1%, $p = 0.355$).

Thromboelastometry studies

The ROTEM studies showed significant differences in the dynamics of clot formation when comparing control and OSA samples. Patients with OSA had an increased alpha angle and MAXV, and an augmented clot firmness assessed by the amplitude at 10 min and MCF. Nevertheless, CT and clot lysis after 60 min was similar in both groups (Fig. 1).

These results showed procoagulant behavior in the hemostasis of the patients with OSA. This clotting test provided integrated information on blood cells and plasma interactions; thus, we attempted to elucidate the role of each of these elements in this thromboelastogram profile.

Functional state of platelets from patients with obstructive sleep apnea

Platelets from patients with OSA were not activated in basal conditions, and their ability to respond to agonists (as evaluated

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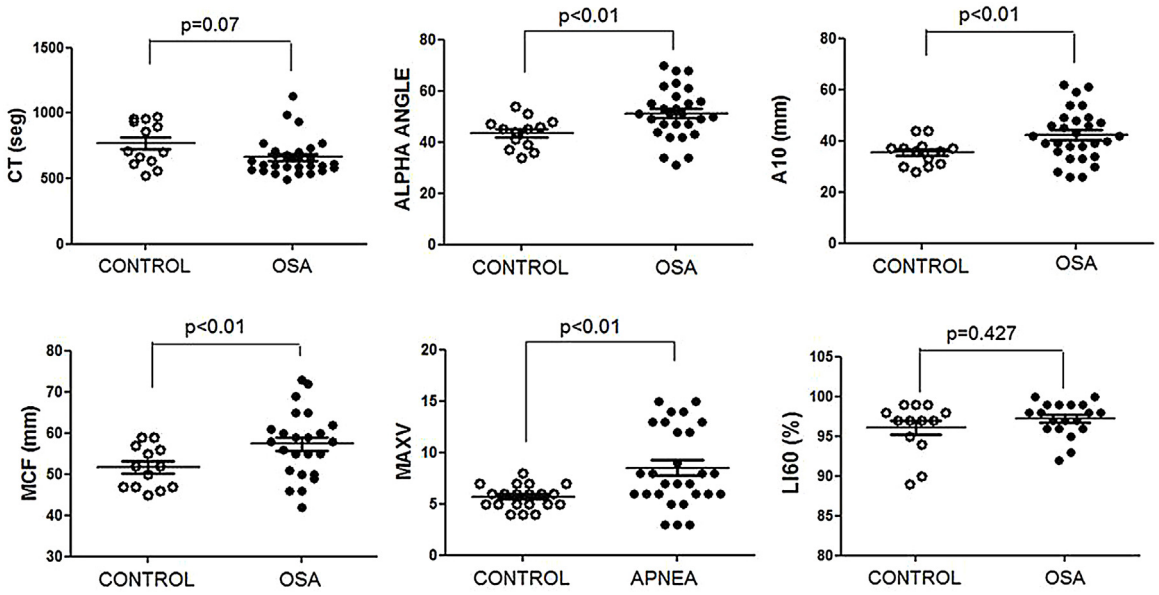

Fig. 1. Features of clot formation in patients with severe OSA. Thromboelastometry was performed in whole blood. CT: time from start of test until 2 mm of amplitude; α angle tangent to the curve at 20 mm amplitude; MCF: maximum clot firmness; MAX_V: maximum velocity of clot formation, LI60: clot lysis at 60 min. The detailed procedure is explained in the Methods section. Mann–Whitney test (for CT analyses) or Student's *t* test was performed, and $p < 0.05$ was considered significant.

by the activation capacity of fibrinogen receptor and of the release of granules' content) was similar to that observed in platelets from healthy controls. Moreover, the surface exposure of receptors for adhesion ligands was the same in both groups (data not shown).

Plasma and microparticle-associated procoagulant activity in patients with obstructive sleep apnea

Fig. 2A shows the thrombin generation (CAT parameters) obtained with PFP samples from the controls and the patients with OSA. Increased peak of thrombin generation was observed in the patients with OSA, indicating that plasma from these patients had a procoagulant profile independent from the procoagulant capacity of MP.

Thrombin generation associated with the TF (MP-Reagent) and PS (PRP-Reagent) content of MPs was also determined. As observed in Fig. 2B and C, patients with OSA had increased thrombin generation related to MPs. Moreover, this effect appeared to be predominantly linked to the TF content of MPs.

The plasma content of nucleic acids might contribute to the creation of prothrombotic profiles. We observed that plasma from patients with OSA had increased free nucleic acids (Fig. 3A).

Plasma levels of E-selectin in patients with severe obstructive sleep apnea

Given TF-rich MPs might originate from endothelial cells as a consequence of endothelial damage, plasma concentration of E-selectin, a marker of endothelial injury, was determined. E-selectin was increased in samples from patients with OSA (Fig. 3B). Moreover, E-selectin plasma levels correlated with low nocturnal SpO_2 (Fig. 3B).

Formation of leukocyte-platelet aggregates in patients with severe obstructive sleep apnea

Patients with OSA formed more leukocyte-platelet aggregates than healthy controls in basal conditions and after stimulation with TRAP and with ADP (Fig. 4).

Relationship between clinical manifestations of obstructive sleep apnea and procoagulant profile

Enhancement of thrombin generation related to plasma as well as to MPs from patients with OSA appeared to be related to the severity of the disease. Patients with the highest AHI presented the most severe procoagulant profile (Fig. 5A). On the other hand, the minimum mean nocturnal and low nocturnal levels of SpO_2 were associated with the highest amount of thrombin generated by plasma (Fig. 5B) and MPs (Fig. 5C).

Discussion

The present study shows that thrombin generation and blood clotting capacity were increased in patients with severe OSA without comorbidities, even in the absence of a previous history of thrombosis. Additionally, we found significant correlations between parameters of thrombin generation and AHI, mean nocturnal SpO_2, and low nocturnal SpO_2, which suggests links between the procoagulant state, and the severity of sleep disorder.

Hypercoagulable features of OSA patients might be due to several factors that can alter hemostasis. For example, it has been reported that patients with OSA have higher hematocrit values, leading to an increase in blood viscosity that correlates with OSA severity.[11] Nevertheless, we did not observe differences in hematocrit values between our cohort of patients with OSA and healthy controls. Moreover, and contrary to Archontogeorgis et al.,[12] we observed similar values in platelet distribution width and mean platelet volume (MPV) between control subjects and apneic patients. These findings suggest that neither hematocrit nor platelet width nor MPV contributed to the higher clotting capacity observed in patients with OSA.

Another factor that could influence the kinetics of clot formation is the platelet activation state and count. However, in our cohort of OSA patients, platelets were not activated in quiescent conditions and had a similar ability to be activated by agonists as those from healthy controls. Our results are in contrast with a previous by von Kanel and Dimsdale,[13] which analyzed five studies on platelet activity in patients with OSA. Although three studies showed higher platelet activation in these patients compared with

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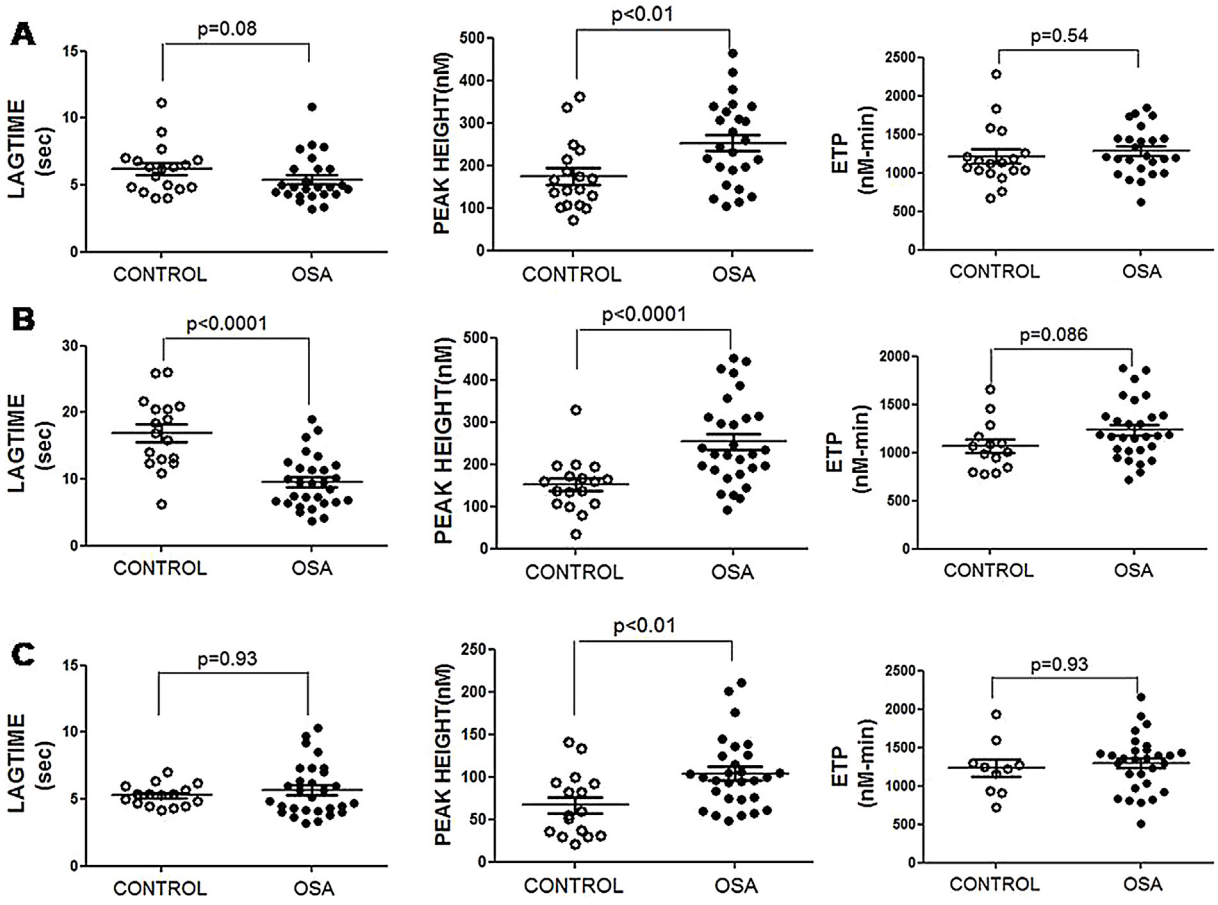

Fig. 2. Plasma- and MP-associated procoagulant capacity in patients with severe OSA. Procoagulant capacity was measured by CAT in plasma samples triggered by the following reagents: PPP-Reagent LOW (A), MP-Reagent (B), and PRP-Reagent (C) to evaluate, respectively, plasma, tissue factor (TF)-associated and phosphatidylserine (PS)-associated procoagulant activity of MPs. Mann–Whitney test (for Lagtime-PPP, and Peak height-PRP analyses) or Student's *t* test was performed, and $p < 0.05$ was considered significant.

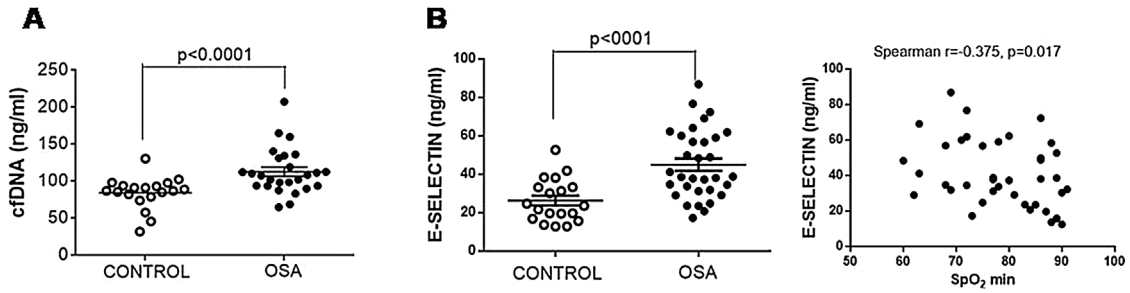

Fig. 3. E-selectin and cfDNA in plasma from patients with severe OSA. (A) Free nucleic acids (cfDNA) in plasma by a fluorometric assay. (B) E-selectin was determined in plasma samples by ELISA. The relationship between plasma levels of E-selectin and low nocturnal SpO_2 is shown. Mann–Whitney test and Spearman's test were performed, and $p < 0.05$ was considered significant.

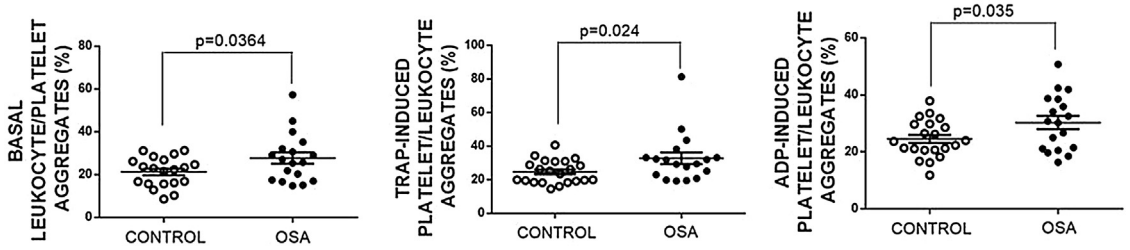

Fig. 4. Leukocyte/platelet aggregates in controls and patients with severe OSA. Leukocyte/platelet aggregates in whole blood, in basal conditions, and after stimulation with 20 μM TRAP or 10 μM ADP. Data are expressed as % of leukocytes positive for αIIb. Mann–Whitney test (for analyses of basal and TRAP-induced aggregates) or Student's *t* test was performed, and $p < 0.05$ was considered significant.

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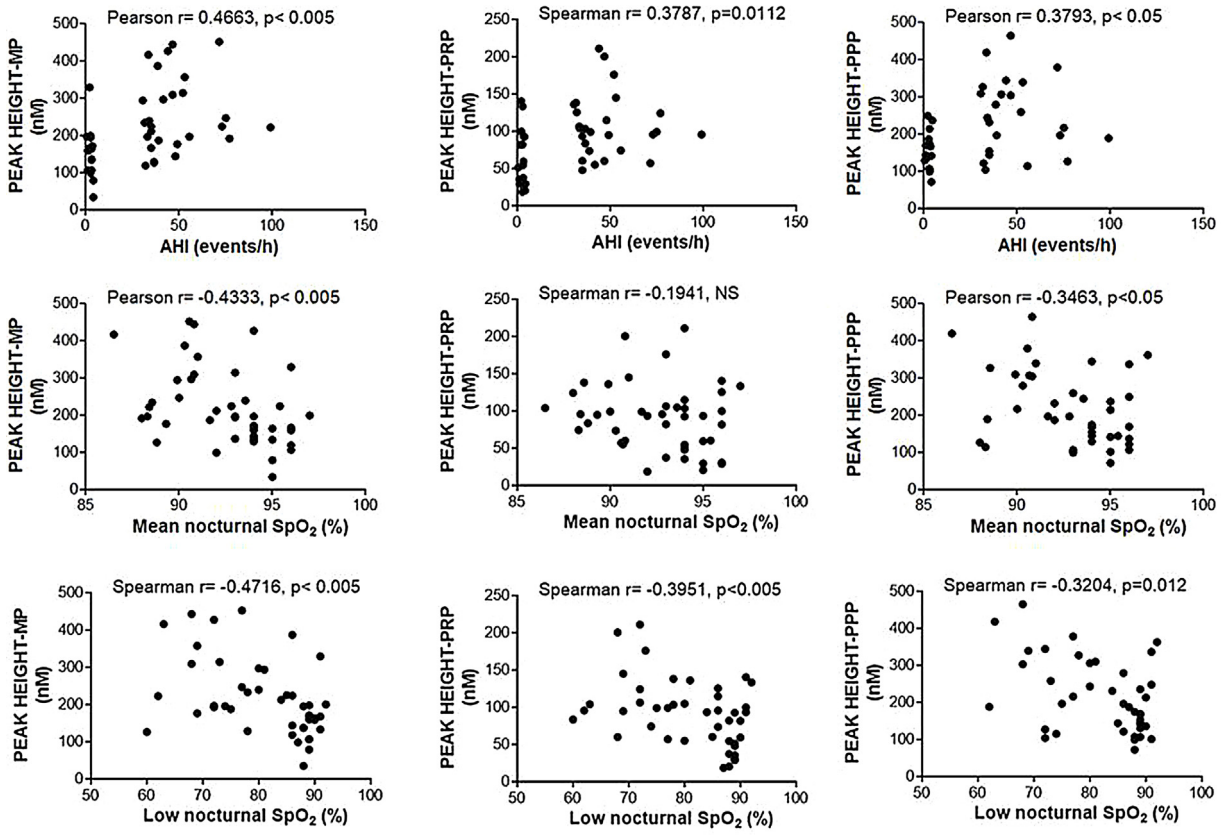

Fig. 5. Correlation between clinical parameters and peak of thrombin generation. Thrombin generation was triggered with either MP- or PRP- or PPP-LOW reagents. Correlation between Peak and: (A) apnea-hypopnea index (AHI), (B) mean nocturnal SpO_2, and (C) low nocturnal SpO_2 are shown. All participants (controls and severe OSA patients) were included in the analysis. Data was analyzed with Spearman's (for analyses of correlations with either Peak height-PRP or Low nocturnal SpO_2) or Pearson's test, and $p < 0.05$ was considered significant.

controls, only one of them provided the statistical signification of this difference. Moreover, another sign of the lack of basal activity in platelets from our cohort of patients with OSA is that MPV, a marker of increased platelet activity,[14] was similar to that observed in the healthy control group.

Regarding the ability of platelets from patients with OSA to be activated by agonists, opposite results have also been reported. Our results and those from West et al.[15]demonstrated that platelets from OSA patients and healthy controls had similar responses to agonist stimulation. On the other hand, Alkhiary et al. reported an increased ADP-induced aggregation of platelets from these patients.[16] However, since a more comprehensive description of their patients is not available, the presence of comorbidities that favor platelet activation cannot be excluded. Even other authors [17]have reported that OSA patients have a decreased platelets aggregation after stimulation with epinephrine but not with ADP or TRAP. All these controversial results on platelet activity and ability to be activated might rely on the characteristics of the cohort of patients as well as on the techniques employed for measuring platelet activation.

However, we observed that patients with OSA presented more platelet/leukocyte aggregates in basal conditions even though they did not have basally activated platelets. This situation can be explained because chronic intermittent hypoxia leads to the production of proinflammatory mediators, such as IL-1β, IL-6, and tumor necrosis factor-α,[18] and inflammatory stimuli are able to increase the formation of platelet-leukocyte complexes in whole blood [19]without inducing platelet aggregation.[20] These results support the hypothesis of a dichotomy in platelet activation depending on the inflammatory versus hemostatic nature of the stimulus.[21]

Other important finding of our study is the identification that plasma from severe OSA patients has increased thrombin generation potential. In contrast, von Kanel et al. found no differences in thrombin–antithrombin and d-dimer (hypercoagulability markers) between patients with OSA and non-OSA controls.[22] However, we believe our results are strong because we measured thrombin generation by a direct technique and not through its degradation products.

Increased clot strength in samples from patients with OSA might also rely on an augmented presence of circulating MPs. Although we did not measure levels of MPs by flow cytometry, we can assume they were increased in our cohort of patients because we observed an enhancement in thrombin generation associated with PS- and TF-containing MPs, and a correlation between their functional activity and their number has been reported.[23] In fact, it has been reported that MPs from monocytes and endothelial cells are the richest TF-containing MPs. Moreover, elevated level of circulating endothelial MPs have previously been reported in patients with OSA without other comorbidities.[24] MPs from monocytes are also considered one of the most thrombogenic MPs.[25] They can be captured by activated platelets within thrombi through a P-selectin/P-selectin glycoprotein 1-dependent mechanism that increases fibrin deposition [26]and docks on endothelial cells, inducing their activation and apoptosis. Along these lines, El Solh et al. demonstrated that patients with OSA have an enhanced apoptosis of endothelial cells that is strongly correlated with soluble TF.[27]

We observed that patients with severe OSA had increased levels of cfDNA in plasma, another factor that can induce a procoagulant profile.[28] CfDNA is released from necrotic and apoptotic cells such as endothelial cells upon activation by proinflammatory

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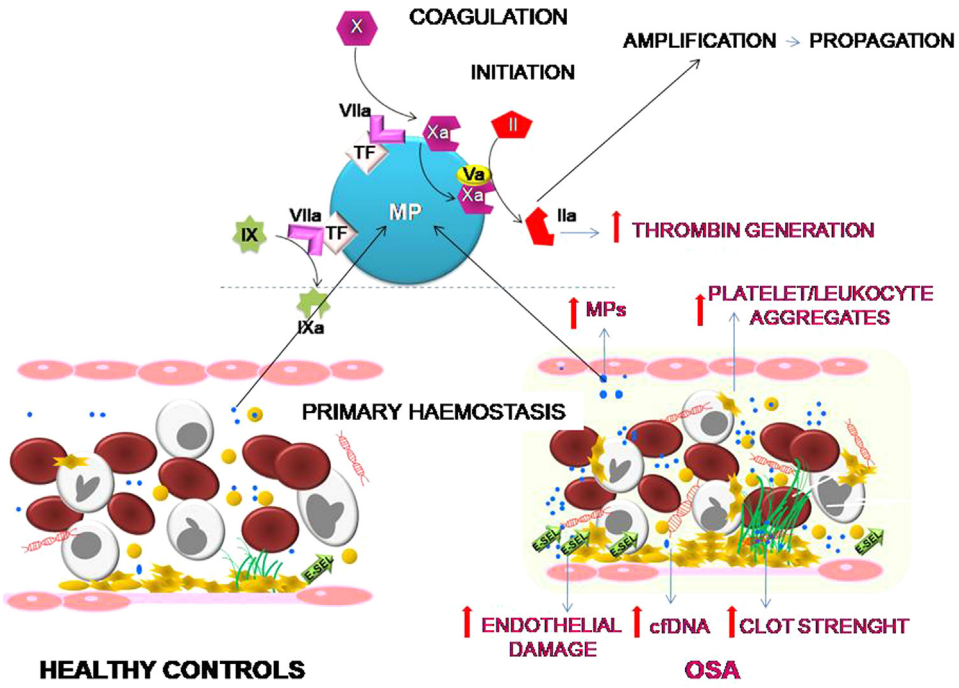

Fig. 6. Effects of OSA on primary hemostasis and coagulation. OSA induces the formation of a stronger clot and increased formation of platelet-leukocyte aggregates. Moreover, OSA provokes an endothelial damage that causes a higher release of cfDNA and of MPs containing TF that enhance capacity for the generation of thrombin.

cytokines. So, cfDNA can be considered a good marker of endothelial damage.[29] Similar results were observed by other authors.[30,31] Elevated plasma levels of E-selectin observed in our cohort of patients confirmed endothelial damage in these patients with OSA. Moreover, we observed that E-selectin levels inversely correlated with nocturnal SpO_2, confirming the relationship between hypoxia and endothelial noxa. Moreover, Cofta et al. reported that soluble E-selectin is the most sensitive indicator of cardiovascular risk among other selectins tested, given it progressively increased severity of OSA.[32]

Correlation analyses of our results have shown associations between AHI, mean nocturnal SpO_2, and low nocturnal SpO_2 with thrombin generation dependent on either plasma or PS- and TF-associated MPs. These significant associations suggest that procoagulant changes vary with OSA severity. Other authors have reported a relationship between mean nocturnal SpO_2 and fibrinogen in patients with OSA,[33] providing additional evidence of the involvement of the blood coagulation system in the increased risk of cardiovascular events observed in these patients.

To our knowledge, only two studies have previously used global tests to determine the kinetics of clot formation, but in these cases they did not use ROTEM, but another global test (thromboelastography) to assess coagulation in patients with OSA.[34,35] One notable observation was that although patients with OSA reportedly have increased plasma levels of plasminogen activator inhibitor-1 (PAI-1), an inhibitor of fibrinolysis [36]we did not detect differences with controls in lysis' values of ROTEM experiments. Similarly, Toukh et al. observed no changes in this parameter.[35] It is tempting to speculate that these tests are not sensitive enough to detect hypofibrinolytic states, but this hypothesis does not appear to be true because we did observe hypofibrinolysis in patients with immune thrombocytopenia.[37] Another similarity with Toukh experiments was the increased clot strength observed in the patients with OSA and the lack of effect of OSA on clot formation onset time. On the other hand, Guardiola et al. observed a reduction in this time.[34] Differences in these results might be due to differences in the study design. None of these studies included a control group, as ours did. Furthermore, the Toukh study had a crossover design in which each patient served as their own control, whereas Guardiola et al. compared independent groups of patients with OSA.

Our study has several limitations that must be acknowledged. First, it is a small, single-center study focused exclusively on patients with severe OSA, so the results should be extrapolated with caution to other hospitals or levels of severity. Second, although there is no difference in the percentage of subjects with obesity between the two study groups, the BMI of our OSA patients was slightly higher than that of the control subjects, so we cannot categorically exclude any contribution of obesity to the identified findings. Third, our study does not provide any information on the effect of treatment of OSA on coagulation pathways, and therefore no therapeutic recommendations beyond those in place for OSA criteria can be formulated.

Conclusion

Our results suggest that OSA induces an increased procoagulant state characterized by an increase in the dynamics of clot formation although without alterations in fibrinolysis. This state appears to be caused by at least two pathological mechanisms. While there does not seem to be a baseline activation of platelets, they do experience a greater formation of platelet-leukocyte aggregates, which seems more dependent on the OSA-induced systemic inflammation than on the activation of hemostatic stimuli. On the other hand, the plasma of patients with severe OSA shows a greater procoagulant activity, with a greater capacity for the generation of thrombin, which seems to depend mainly on the increase in MPs containing TF that are produced as a consequence of the endothelial damage (Fig. 6). Moreover, this study has identified thromboelastometry and thrombin generation assay as useful tools to evaluate the prothrombotic state in these patients. These techniques might have decision-making potential to guide therapy in patients with OSA.

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Author contributions

IFB, EMM, RJS, PA and CC-Z performed the experiments; RC, BS, AJ, AA-F and FGR recruited patients and collected clinical data; IFB, MTAR, VJY, FGR and NVB analyzed data of work, FGR and NVB designed the study; NVB wrote the manuscript; all authors revised critically the paper and approved it.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.arbres.2020.11.017.

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Platelets

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Pro-apoptotic properties and mitochondrial functionality in platelet-like-particles generated from low Aspirin-incubated Meg-01 cells

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ARTICLE

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Pro-apoptotic properties and mitochondrial functionality in platelet-like-particles generated from low Aspirin-incubated Meg-01 cells

Gala Freixer[1], Khaoula Zekri-Nechar[1], José J. Zamorano-León[2], Carlos Hugo-Martínez[1], Nora V Butta[3], Elena Monzón[3], María-José Recio[4], Manel Giner[5], & Antonio López-Farré[1]

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Abstract

Long-term therapy with low Aspirin (ASA) dose is basis to prevent thrombotic acute events. However, the anti-platelet mechanisms of ASA remain not completely known. The aim was to analyze if in vitro exposure of human megakaryocytes to low ASA concentration may alter the apoptotic features of the newly formed platelets. Cultured Meg-01 cells, a human megakaryoblastic cell line, were stimulated to form platelets with 10 nmol/L phorbol 12-myristate-13-acetate (PMA) in the presence and absence of ASA (0.33 mmol/L). Results revealed that platelet-like particles (PLPs) derived from ASA-exposed Meg-01 cells, showed higher content of pro-apoptotic proteins Bax and Bak than PLPs from non-ASA incubated Meg-01 cells. It was accompanied of reduced cytochrome C oxidase activity and higher mitochondrial content of PTEN-induced putative kinase-1 in PLPs from ASA-incubated Meg-01 cells. However, only after calcium ionophore A23187 stimulation, caspase-3 activity, the cytosolic cytochrome C content, and reduction of mitochondrial membrane potential were higher in PLPs from ASA-incubated megakaryocytes than in those from Meg-01 without ASA. Nitric oxide synthase 3 content was higher in PLPs from ASA-exposed Meg-01 cells than in PLPs from non-ASA incubated Meg-01 cells. The L-arginine antagonist, NG-Nitro-L-arginine Methyl Ester, reduced caspase-3 activity in A23187-stimulated PLPs generated from ASA-incubated Meg-01 cells. As conclusions exposure of megakaryocyte to ASA promotes that the newly generated PLPs have, under stimulating condition, higher sensitivity to go into apoptosis than those PLPs generated from Meg-01 cells without ASA. It could be associated with differences in mitochondrial functionality and NO formation.

KEYWORDS

Apoptosis, Aspirin, Megakaryocytes, Mitochondria, Nitric Oxide, Platelets

History

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Introduction

Platelets have the ability to self-destruct by the intrinsic apoptotic pathway [1]. It is triggered by changes in the mitochondrial integrity, including modulation in the mitochondrial membrane potential ($\Delta\Psi m$) and by the release to cytosol through pores in the mitochondrial membrane of apoptotic cofactors, such as cytochrome C, and the involvement of pro-apoptotic proteins such as Bak and Bax [2,3]. Once released from mitochondria, cytochrome C acts stimulating caspase proteins, mainly caspase-3 activity [4,5]. The intrinsic apoptotic program may be induced in the platelets by long-term platelet store but also by external stimuli such as calcium ionophore [5,6].

Platelets contain mitochondria and mitochondria lifespan determine platelet lifespan. In this regard, mitochondrial homeostasis is tightly regulated to ensure a number of mitochondria with appropriate mitochondrial functionality [7]. Mitochondrial dysfunction occurs following inhibition of the mitochondrial oxidative phosphorylation chain, which decrease ATP production and increase ROS formation. Mitophagy is the mechanism to eliminate dysfunctional mitochondria delivering them into autophagosomes for lysosomal degradation [8]. In the mitochondria, two main proteins working together regulate mitophagy, i.e. PTEN-induced putative kinase 1 (PINK-1) and Parkin proteins [9].

Several trials have suggested that long-term therapy with low daily Aspirin (ASA) dose is effective to prevent arterial and venous thrombotic events [10,11]. The main known mechanism associated with reduction of the platelet activity by ASA is the irreversible inhibition of platelet cyclooxygenase (COX)-1 activity, thereby preventing platelet generation of thromboxane A_2 (TxA_2) [12]. A second well established mechanism to explain the antiplatelet effects of ASA is the stimulation of nitric oxide synthase 3 (NOS3) to produce nitric oxide (NO), a potent inhibitor of platelet activation [13,14].

Besides to inhibit platelet aggregation, in the platelets ASA may also alter the level of several proteins involved in the platelet functionality. As example, we recently reported that ASA responders platelets from patients under chronic treatment with low ASA dose showing differences in the level of expression of not secretable proteins such as the NOS3 protein and proteins involved in platelet metabolism and oxidative stress [15,16]. Since mature platelets have a diminished ability to synthesize new proteins, these observations clearly open the possibility that ASA may acts on megakaryocytes to promote the generation of newly platelets showing changes in the level of some proteins contained in them.

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It has been previously examined the effect of high ASA concentrations, anti-inflammatory concentrations, on platelet apoptosis. In animals models an in in vitro incubated human platelets these anti-inflammatory ASA concentrations seem to reduce platelet lifespan [17,18]. However, in our knowledge, it has not been explored if low ASA concentration, similar to that reached in the plasma of patients underwent to long-term daily ASA-treatment to prevent acute thrombotic events, may be acting on megakaryocytes to promote production of newly platelets with a different apoptotic profile. Therefore, the aim of the present work was to study if the in vitro incubation of human megakaryocytes with low ASA concentration, similar to that achieved in long-term ASA-treated patients, may influence the apoptotic features of the newly generated platelets.

Methods

Meg-01 Culture

Experiments were performed using the Meg-01 cells (Sigma, St Louis, MO. USA), an established human megakaryoblastic leukemia cell line extensively used as model of human megakaryocytes. Therefore, in the text was equally used the terms Meg-01 cells and megakaryocytes.

In response to stimuli, cultured Meg-01 release platelets that have been defined as platelet-like particles (PLPs) [19]. The PLPs generated from stimulated Meg-01 have been reported to share some similarities with human platelets [20,21].

For the experiments, Meg-01 cells (1×10^4 cells) were seeded in a well of 6-well plate and grown in RPMI 1640 medium (GIBCO) supplemented with 10% fetal calf serum (FCS), 5 mmol/l L-glutamine and antibiotics at 37°C and 5% CO_2 atmosphere. Before starting the experiments, Meg-01 cells were synchronized by growing in 0.5% FCS during 24 h. To accelerate the formation of PLPs from Meg-01 cells, Meg-01 cells were incubated with 10 nmol/L phorbol 12-myristate-13-acetate (PMA) for 72 hours. In some experiments, acetylsalicylic acid (ASA, 0.33 mmol/L, Sigma Aldrich, St Louis, MO) was added together with PMA. This ASA concentration was similar to that calculated from measurements of serum salicylic acid in volunteers daily taking low dose of ASA (200 mg/day) [13]. Meg-01 cells were used within 10 passages from the original Meg-01 cells purchased from the commercial distributor.

As reported [21–23], 72 hours after PMA addition, preparations were centrifuged at 150 g for 10 minutes. The sediments were discarded and the supernatant was centrifugated at 800 g for 15 min. The obtained supernatants were again centrifugated at 1600 g for 15 minutes and sediments containing PLPs were recovered. PLPs were counted using a Scepter 2.0 automated cell counter (Ref PHCC20060. Merck Millipore. Darmstadt. Germany).

Determination of Surface Receptor in PLPs by Flow Cytometry

Previous works have studied properties of PLPs [20,21]. However, we also determined the plasma membrane localization of platelet-associated proteins comparing PLPs with human platelets by using flow cytometry.

Circulating platelets were obtained from a healthy volunteer that had not taken any anti-thrombotic or anti-inflammatory drug for at least 15 days before blood extraction. The protocol had been approved by the local Ethics Committee (C.I 12/163-E) and it was performed according with the 1964 Helsinki declaration. As previously reported [24] PLPs and human platelets were incubated in the darkness for 20 min at room temperature with the following antibodies: fluorescein (FITC)-labeled monoclonal anti-CD42b antibody (BD Pharmingen, San Diego, CA, USA); phycoerythrin (PE)-labeled monoclonal anti-CD41 antibody (BioCytex, Marseille, France); and FITC-labeled monoclonal anti-*P*-selectin antibody (BD Pharmingen, San Diego, CA, USA). Flow cytometry was performed using the CMF, FACScan flow cytometer, BD Bioscience, Madrid, Spain). For each analysis, 5000 events were acquired and data were analyzed by the BD CellQuest Pro TM software (Becton Dickinson Biosciences).

Western Blot Analysis

The level of expression of different proteins was evaluated by Western blot as we previously reported [25,26]. For this purpose, cells, were homogenized in a lysis buffer containing 8 mol/L urea, 2% CHAPS w/v, 40 mmol/L dithiothreitol. About 40 µg of total PLPs proteins, estimated by bicinchonioic acid reagent (Pierce), were developed in 15% SDS/PAGE.

Nitrocellulose membranes were incubated with monoclonal antibodies against Bak (1:1000; Y164, Abcam. Cambridge UK) and NOS3 (sc-653, Santa Cruz Biotechnology Inc. CA) and polyclonal antibodies against Bax (1:1000; PC66, Oncogene Research Products. La Jolla, CA), mitochondrial Mn-superoxide dismutase (SOD2; 1:1000; sc-30080, Santa Cruz Biotechnology, Inc. CA) and NADPH oxidase subunit gp91-phox (1:1000; sc-5827 Santa Cruz Biotechnology Inc). A monoclonal anti-β-actin antibody (1:7500, A-5441, Sigma-Aldrich, St. Louis, MO, USA) was used as a control for protein loading. Nitrocellulose membranes were then revealed with peroxidase-conjugated anti-rabbit IgG (1:2500) for SOD2, NOS3, and Bax; peroxidase-conjugated anti-goat IgG antibody (1:2500 for gp91-phox, and Bak) and peroxidase-conjugated anti-mouse IgG (1:7500 for β-actin). The protein signal was developed by chemiluminescence (ECL; GE Healthcare, Little Chalfont Buckinghamshire, UK) and detected using the iBright Imaging System (iBright FL100, Thermofisher Scientific Massachussetts, USA).

Mitochondria and Cytosol Isolation: cytosolic Cytochrome C Level Determination

Cytosolic and mitochondrial extracts from the PLPs were obtained using a commercial mitochondria isolation Kit (catalog#8987, Thermoscientific, IL, USA) that allows mitochondria and cytosol isolation and it was carried out following manufacturer's specifications.

To identify cytosolic cytochrome C content, and due to the relative low level of cytosolic cytochrome C in platelets, we could not use the Western blot technique. Therefore, cytochrome C content in the isolated cytosols was detected with dot-blot analysis. In brief, to perform dot-blot analysis 2.5 µg of total cytosolic proteins was loading onto nitrocellulose membranes [25,26]. Nitrocellulose membranes were then blocked with 5% (w/v) bovine serum albumin and, after washed, were incubated with a polyclonal cytochrome C antibody (1:1000 AB90529, Abcam. Cambridge UK). Then, nitrocellulose membranes were incubated with a peroxidase-conjugated anti-mouse IgG and developed using chemiluminiscence and detected by the iBright Imaging System.

Caspase-3 Activity

To determine caspase-3 activity, 250 µg platelet protein/sample was used. Caspase-3 activity was measured using a colorimetric commercial kit (ab39401, Abcam, Cambridge, UK). The colorimetric assay kit was based on the detection of the chromophore *p*-nitroaniline and it was performed following the manufacturer's specifications. As manufacturer recommended, caspase-3 activity

was expressed as Optical Density (O.D.) units at 405 nm after 120 min incubation.

Changes in Mitochondria Membrane Potential (Δψm)

ΔΨm was assessed using a JC-10 fluorometric assay kit (MAK159 Sigma-Aldrich. St Louis, MO. USA) according to the manufacturer's protocol. In brief, JC-10 dye loading solution (final concentration 2 μg/ml) was added to 5×10^5 PLPs/well that were incubated in the dark for 45 min, at 37ºC. Before fluorescence measurement in some experiments A23187 (10 μmol/L) was added to PLPs.

Red and green fluorescences were detected using the iBright Imaging System. Red fluorescent (JC-10 aggregates) at 540 nm excitation and 590 nm emission, while green fluorescent (JC-10 monomers) were detected at 490 nm excitation and 525 nm emission. The ratio of fluorescence intensities at 590 nm/525 nm was used to determine mitochondrial membrane depolarization that was considered by reduction in the red to green fluorescence intensity ratio.

Citrate Synthase and Cytochrome C Oxidase Activities in PLPs

Citrate synthase activity un PLPs was determined using a colorimetric commercial kit (CS0720, Sigma-Aldrich, St Louis, MO. USA) following the manufacturer's specifications. Citrate synthase determination was based on the conversion of acetyl-CoA and oxalacetic acid into a thiol group (CoA-SH) that reacts with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to form 5-thio-2-nitrobenzoic acid (TNB) that is detected at 412 nm.

Cytochrome C oxidase activity was also measured using a specific colorimetric commercial kit (109911. Abcam. Cambridge UK). The assay kit was based on the oxidation of reduced cytochrome C at 550 nm and it was carried on following the manufacturer's instructions.

Mitochondrial Content of PTEN-Induced Putative Kinase 1 (PINK-1) and PARKIN

Isolated mitochondria from PLPs were lysates in the above described lysis buffer and centrifuged at 12,000 g for 10 min. As platelets contain a very low mitochondria content, dot-blot analysis was performed. The nitrocellulose membranes were incubated with an anti-PINK-1 polyclonal antibody (1:1000; ab23707. Abcam. Cambridge UK) or an anti-Parkin antibody (1:1000; ab15954, Abcam). After that, membranes were incubated with a peroxidase-conjugated anti-rabbit IgG and developed using chemiluminescence reagents (ECL; GE Healthcare, Little Chalfont Buckinghamshire, UK) and detected by the iBright Imaging System (Ibright FL100, Thermofisher Scientific. Massachussetts, USA).

Statistical Analysis

Values are expressed as mean ± standard error of mean (S.E.M). The statistical analysis was performed using the Mann–Whitney test. To analyze the concentration-response effects of ASA and salicylic acid on the expression of pro-apoptotic proteins the Wilcoxon test was used comparing each specific concentration with the experiments performed in the absence of ASA or salicylic acid. The SPSS 22.0 software was used and a p value <.05 was considered as statistically significant.

Results

PLPs Characteristics and Comparison with Circulating Human Platelets

Meg-01 cells were markedly larger in size (forward scatter) than either PLPs or human circulating platelets (Figure 1). Accordingly, it also supports the purity of the isolated PLPs.

Comparison of size and complexity between the newly generated PLPs and human platelets showed that many of the PLPs had similar values of size and complexity as platelets but PLPs population showed a significant more heterogeneous distribution than human platelets (Figure 1). It may be in accordance with previous reported observations [27,28]

Although Meg-01 cells have been widely used as in vitro model to study the formation and features of the newly formed particles [20,23], we determined the presence of some platelet-related plasma membrane antigens in these PLPs.

Taking the entire PLPs population, proportion of plasma membrane CD41 (GPIIbα)-positive PLPs were 16% while in circulating human platelets CD41 positive was 96.00%. Percentage of plasma membrane CD42b (GPIb)-positive PLPs were only 1.84% while 92.22% of human platelets were positively labeled for CD42b. These findings are in accordance with some previous reports, although other works have reported higher percentage of positivity for these antigens in PLPs but in these studies only proportion of PLPs similar in size to platelets were considered and not the entire PLPs population as we did in the present work [21,29,30]

To analyze the spontaneous activation of PLPs by the isolating manipulation, the surface expression of CD62 antigen was also measured. Proportion of positive PLPs for CD62 antigen was very low and there were no differences between PLPs generated from ASA-incubated and ASA-non incubated Meg-01 cell (-ASA: 1,84%, +ASA: 2,68% pNS). This suggests a very low activation of PLPs by the methodology used for its isolation. In all cases of the identified platelet surface proteins, unstained control represented less than 0.30%.

Expression of Pro-apoptotic Proteins in the Newly Formed Platelets

Western blot experiments showed that the newly PLPs generated from ASA-incubated Meg-01 cells had higher content of the pro-

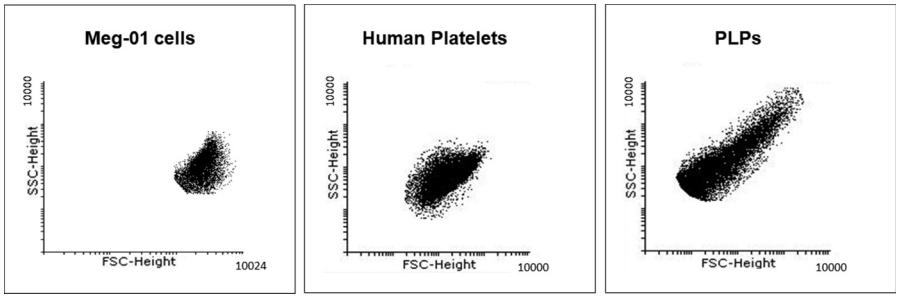

Figure 1. Size and complexity of Meg-01 cells, platelet-like particles (PLPs) and human platelets.

Flow cytometry dot plots show complexity (Side Scatter, SSC) and size (Forward Scatter, FSC) of PLPs, human platelets and Meg-01 cells. Human platelets were obtained by venipuncture from a healthy volunteer.

apoptotic Bak protein, than PLPs from non-ASA incubated megakaryocytes (Figure 2A). In the PLPs, a significant increase of Bak protein was already observed from 0.33 mmol/L ASA-incubated Meg-01 cells (Figure 2A), a similar ASA concentration to that detected in the serum of patients underwent to low ASA treatment (200 mg/day)[13]. The following experiments were then performed using 0.33 mmol/L ASA.

The major route of elimination of ASA is salicylic acid. The presence of salicylic acid on Meg-01 cells also increased Bak content in the newly formed PLPs (Figure 2A). However, the salicylic acid concentration needed to increase the content of Bak in the newly formed PLPs was higher than those for ASA (Figure 2A). Indeed, incubation of Meg-01 cells with 0.33 mmol/L salicylic acid did not significantly modified Bak content in the newly formed PLPs (Figure 2A). In the PLPs a significant increase in Bak protein content was reached when Meg-01 cells were incubated with 0.66 mmol/L salicylic acid (Figure 2A).

The content of the pro-apoptotic Bax protein was also increased in the PLPs generated from 0.33 mmol/L ASA-incubated Meg-01 cells as compared with those from Meg-01 cells incubated without ASA (Figure 2B). An interesting observation was that 0.33 mmol/L ASA slightly but significantly increased Bak content in the Meg-01 cells themselves with respect to Meg-01 cells without ASA (Figure 2C).

As Figure 2D shows, the number of PLPs generated from PMA-stimulated Meg-01 cells incubated with ASA was slightly but significantly higher as compared with those PLPs generated from PMA-stimulated Meg-01 cells without ASA.

Despite of the fact that Bax and Bak protein expression was higher in PLPs from ASA-incubated Meg-01, however, neither cytosolic content of cytochrome C nor caspase-3 activity were different between PLPs from Meg-01 incubated with and without ASA (Figure 3A and B). In this regard, at basal conditions cytosolic cytochrome C levels were almost undetectable independently that PLPs were generated from either Meg-01 incubated in the presence and in the absence of ASA (Figure 3A).

Effect of Platelet Stimulation on the Apoptotic Status

Despite of the apparently paradoxical results where PLPs from ASA-incubated Meg-01 cells had higher content of two pro-apoptotic proteins but it had been not reflected on changes of apoptotic-associated pathways, we then studied if the apoptotic state could be modified under conditions of platelet stimulation. PLPs were then incubated with calcium ionophore (10 µmol/L) at 37ºC during 120 min. After A23187 stimulation, PLPs generated from Meg-01 incubated in the presence and in the absence of ASA showed higher cytosolic cytochrome C content and caspase-3 activity than their respective basal conditions (Figure 3A and B). However, after A23187 stimulation, PLPs from ASA-incubated Meg-01 cells showed significant higher increase of both caspase-3 activity and cytochrome C levels in their cytosol than that detected in the cytosol of A23187-stimulated PLPs generated from non-ASA-incubated Meg-01 cells (Figure 3A and B).

The fact that higher sensitivity to apoptosis in A23187-stimulated PLPs was associated with the previous presence of low ASA concentration in Meg-01 cells during PLPs formation was also supported by the changes in $\Delta\Psi m$. In this regard, Table 1 shows at basal conditions $\Delta\Psi m$ was significantly higher in

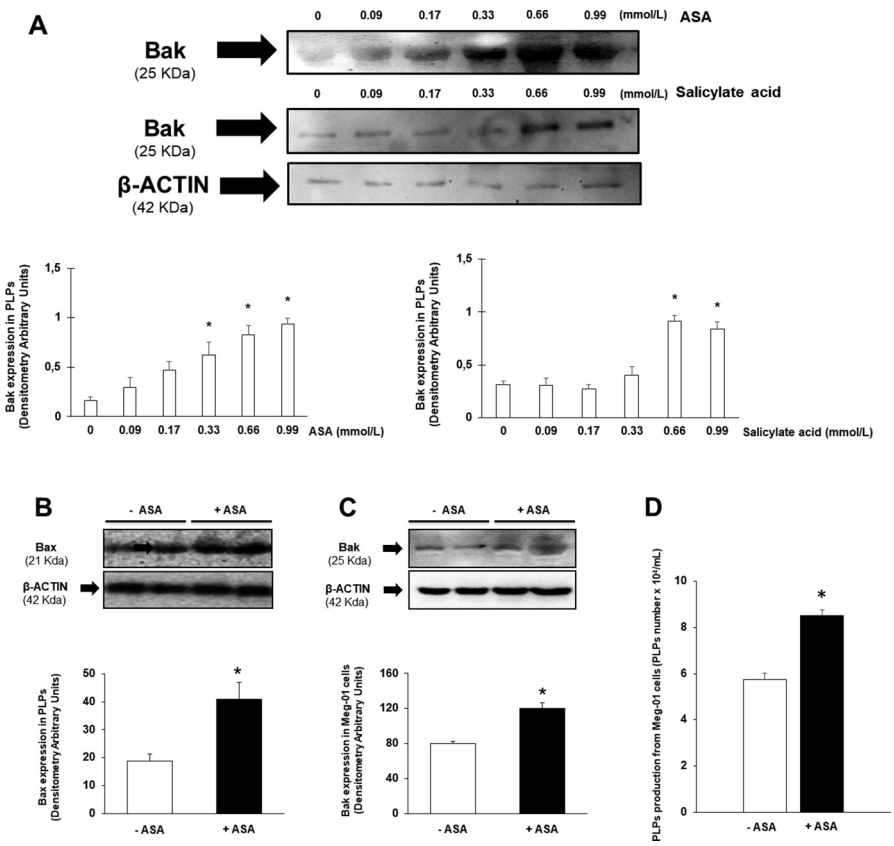

Figure 2. Expression of pro-apoptotic proteins Bak, Bax in the newly formed platelet-like particles (PLPs) and in Meg-01 cells.

A: Representative Western blots show the content of Bak protein in PLPs generated from Meg-01 cells. Meg-01 cells were incubated under increased ASA or salicylate acid concentrations. β-actin expression was used as protein loading control. Bar graphs show the densitometry analyses of all the Western blots represented in arbitrary units (A.U.). Results are represented as mean ± SEM of three independent experiments from three different Meg-01 passages. **B: Upper Left**: Representative Western blots showing the Bax content in PLPs generated from Meg-01 cells incubated with and without 0.33 mmol/L ASA. β-actin expression was used as loading protein control. **Bottom**: Bar graph shows the densitometry analyses of four independent experiments from different Meg-01 passages. **C: Upper Right**: Representative Western-blots showing the expression of Bak protein in Meg-01 cells incubated in the presence and in the absence of 0.33 mmol/L ASA. β-actin expression was used as loading protein control. **Bottom**: Bar graph represents the densitometry analysis of three independent experiments performed in Meg-01 cell from different passages. Results are represented as mean ± SEM. **D**: Bar graph shows the number of PLPs generated from PMA-stimulated Meg-01 cells (1×10^4 cells) incubated with and without ASA. Results are represented as mean± SEM of three independent experiments in PLPs from Meg-01 cells of different passages. * $p < .05$ respect to the experiments performed without ASA. * $p < .05$ respect to the experiments performed without ASA or salicylate acid.

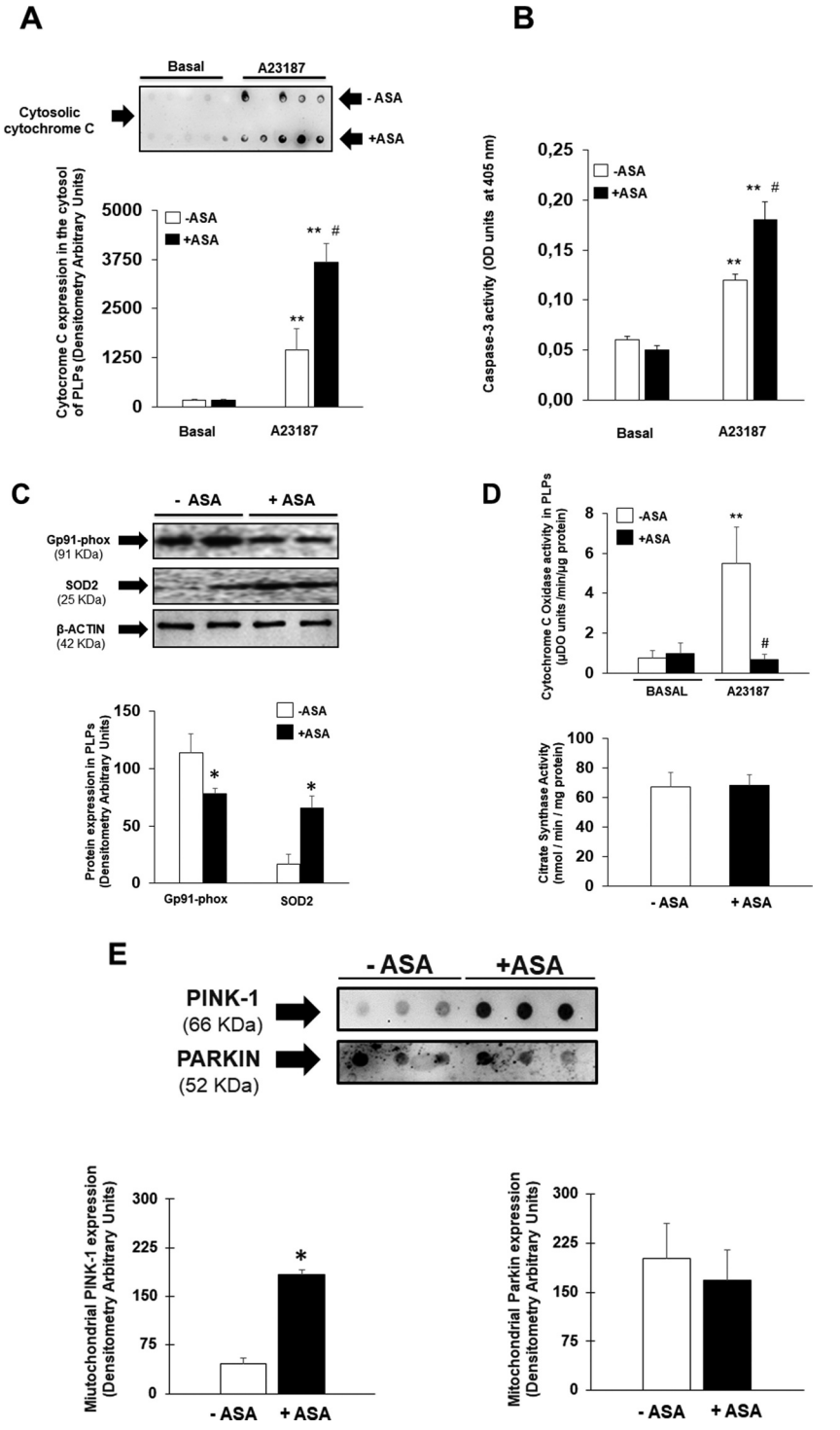

Figure 3. Cytosolic content of cytochrome C and caspase-3 activity in the newly formed platelet-like particles (PLPs).

A: Upper: Representative dot-blot showing cytochrome C content in the cytosol of PLPs generated from ASA-incubated and ASA-non-incubated Meg-01 cells. Experiments with calcium ionophore A23187 (10 μmol/L) were also performed. **Bottom**: Bar graphs shown the densitometry analysis of twelve independent experiments from different passages of Meg-01 cells. **B**: Caspase-3 activity in PLPs formed from Meg-01 cells incubated with and without ASA. Experiments with calcium ionophore A23187 (10 μmol/L) were also performed. The mean ± SEM of five independent experiments in PLPs from different Meg-01 passages are represented. **C: Upper**: Representative Western blots showing the content of mitochondrial gp91-phox NADPH oxidase subunit and Mn-superoxide dismutase isotype 2 (SOD2) in PLPs generated from Meg-01 cells incubated in the absence and in the presence of 0.33 mmol/L ASA. β-actin expression was used as protein loading control. **Bottom**: Bar graph shows the analyses of all the Western blots represented in densitometry arbitrary units (A.U.). Results are represented as mean ± SEM of four independent experiments from different Meg-01 cells passages. **D: Upper**: Bar graph shows the enzymatic activity of mitochondrial cytochrome C oxidase in PLPs generated from Meg-01 cells incubated in the absence and in the presence of ASA. PLPs were stimulated or not with 10 μmol/L calcium ionophore A23187. Results are represented as mean ± SEM of five independent experiments. **Bottom**: Bar graph represents citrate synthase activity in PLPs generated from Meg-01 cells incubated with and without ASA. Results are represented as mean ± SEM of four independent experiments in PLPs from Meg-01 cells of different passages. **E: Upper**: Representative dot-blot showing the mitochondrial content of PTEN-induced putative kinase 1 (PINK-1) and Parkin proteins. Mitochondria were obtained from PLPs generated from Meg-01 cells incubated in the absence and in the presence of 0.33 mmol/L ASA. **Bottom**: Bar graph showing the densitometry analyses of the performed dot blots represented in densitometry arbitrary units. Results are represented as mean ± SEM of three independent experiments performed in PLPs derived from Meg-01 cells of different passages. * $p < .05$ with respect to PLPs from Meg-01 cells incubated without ASA. ** $p < .05$ with respect to corresponding PLPs at basal conditions. # $p < .05$ with respect to A23187-stimulated PLPs derived from Meg-01 cells incubated without ASA.

Table 1. Changes in Ψm in PLPs.

	– ASA	+ ASA
Basal	1.92 ± 0.28	5.08 ± 0.95 *
+ A23187	1.06 ± 0.34	2.73 ± 0.98 *
ΔΨm variation (Basal – A23187)	0.86 ± 0.06	2.35 ± 0.36 *

ΔΨm is represented in densitometry arbitrary units and was calculated as λ emission ratio 590 nm/525 nm. Results are expressed as mean ± SEM of three independent experiments in PLPs derived from different Meg-01 passages. *$p < .05$ respect to PLPs generated from Meg-01 cells incubated without ASA.

PLPs generated from Meg-01 cells incubated with ASA. The presence of A23187 (10 μmol/L) significantly reduced the ratio 590 nm/525 nm in both groups of PLPs although it was more evident in PLPs derived from ASA-incubated Meg-01 cells (Table). Then, ΔΨm variation between at baseline and after A23187 stimulation was significantly higher in PLPs from ASA-incubated Meg-01 cells than that in PLPs generated from Meg-01 cells incubated without ASA (Table 1). This observation again supported the hypothesis that in stimulating conditions higher apoptosis is promoted in PLPs derived from ASA-incubated Meg-01 cells than in PLPs from Meg-01 cells without ASA.

Mitochondrial Content of Proteins Associated with Oxidative Stress

Mitochondrial reactive oxygen species may act as mediators of platelet apoptosis. The expression of the mitochondrial gp91-phox NADPH oxidase subunit was lower in PLPs from ASA-incubated Meg-01 cells than in those PLPs generated from megakaryocytes incubated without ASA (Figure 3C). Moreover, the content of the mitochondrial superoxide dismutase (SOD) isoform 2, the SOD2, was higher in PLPs generated from ASA-incubated megakaryocytes than in those PLPs generated from Meg-01 cells incubated without ASA (Figure 3C).

Mitochondrial Functionality in the Newly Formed PLPs

At basal conditions, cytochrome C oxidase activity, a key-stop enzyme of the mitochondrial phosphorylation chain, was similar between PLPs from Meg-01 incubated in the presence and in the absence of ASA (Figure 3D). However, when A23187 calcium ionophore was added a significant increase of cytochrome C oxidase activity was observed in PLPs generated from Meg-01 cells without ASA (Figure 3D). A23187 did not modify cytochrome C oxidase activity in the newly PLPs formed from ASA-incubated Meg-01 cells, even maintaining values similar to those observed at baseline conditions (Figure 3D).

Citrate synthase activity, enzyme used as marker of mitochondrial density [31], was not different between PLPs derived from ASA-incubated and ASA-non incubated Meg-01cells (Figure 3D).

The mitochondrial content of PINK-1 protein was significantly higher in PLPs generated from ASA-incubated Meg-01 cells as compared with those generated from Meg-01 without ASA (Figure 3E). Mitochondrial content of Parkin protein was similar between PLPs derived from Meg-01 cells incubated in the presence and in the absence of 0.33 mmol/L ASA (Figure 3E).

Possible Involvement of Nitric Oxide on the PLPs Apoptosis Promoted by the Incubation of Meg-01 Cells with ASA

Incubation of Meg-01 cells with 0.33 mmol/L ASA significantly increased NOS3 protein content in the newly formed PLPs with respect to PLPs from Meg-01 cells without ASA (Figure 4). Addition of the L-arginine antagonist, 10^{-4} mol/L NG-Nitro -L-arginine Methyl Ester (L-NAME) 15 min before being stimulated with calcium ionophore A23187, significantly reduced caspase-3 activity in PLPs generated from 0.33 mmol/L ASA-incubated Meg-01 cells (A23187: $190 \times 10^{-3} \pm 10\times10^{-3}$; A23187 + L-NAME: $130 \times 10^{-3} \pm 20\text{x}10^{-3}$; OD units at 405 nm per 100 µg of total PLPs protein in 120 min; n = 3, $p = .039$).

Discussion

The present work shows, in our knowledge for the first time, that human megakaryocytes incubated with low ASA concentration, similar to that achieved in patients daily treated with ASA, produced newly PLPs with higher content of pro-apoptotic proteins Bak and Bax than PLPs generated from Meg-01 cells incubated without ASA. However, only after stimulation of PLPs with calcium ionophore A23187 either, caspase-3 activity, the cytosolic cytochrome C levels, and the decrease in $\Delta\Psi m$, key markers for apoptotic execution, were significantly higher in PLPs from ASA-incubated Meg-01 cells than in those PLPs derived from megakaryocytes incubated without ASA. These observations suggested that the presence of ASA in PMA-stimulated megakaryocytes promotes the generation of newly platelets with characteristics for better predisposition to going into apoptosis when a situation of platelet stimulation was produced.

Previous works had demonstrated that anti-inflammatory ASA concentrations, almost 10-fold higher than the here tested, directly promotes apoptosis in the platelets, even under resting conditions [17,18]. Our study provides a novel concept showing that a low

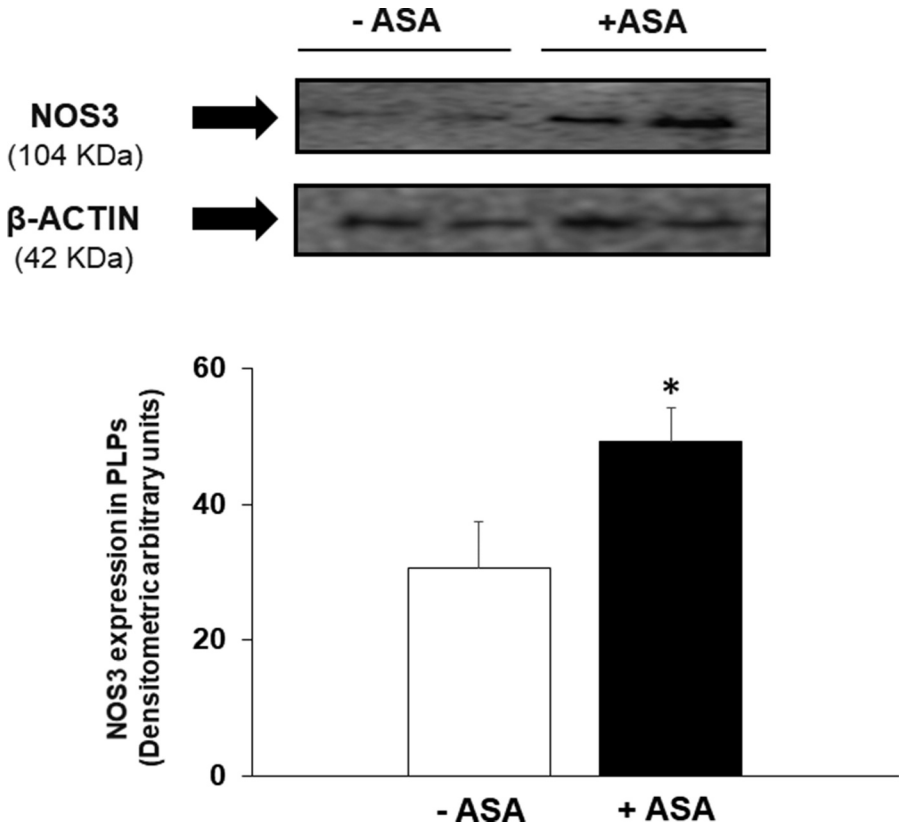

Figure 4. Nitric oxide synthase 3 (NOS3) expression in the newly formed platelets-like platelets (PLPs).

Upper: Representative Western blot showing NOS3 content in PLPs generated from Meg-01 cells incubated in the absence and in the presence of 0.33 mmol/L ASA. β-actin expression was used as loading protein control. **Bottom**: Bar graphs showing the densitometric analyses of all the performed Western blots. The values are represented in densitometry arbitrary units as mean ± SEM of six independent experiments from different Meg-01 passages $*p < .05$ respect to PLPs-derived from Meg-01 incubated without ASA.

ASA concentration similar to that achieved in patients under chronic vascular ASA treatment, acting on megakaryocytes may modify the apoptotic ability of the newly platelets generated from them.

The ASA concentration in which significant changes were archived on in the platelet content of Bax and Bak proteins was similar to that found in the serum of healthy volunteers underwent to 200 mg/day ASA treatment during 4 days. It is important to point out that in that work, [13] ASA measurements were performed by determining in serum the main acetylsalicylic metabolite, salicylic acid, that was then calculated in equivalent concentration, on the molar basis, to serum acetylsalicylic acid concentration. It results in an ASA concentration of 0.33 mmol/L. However, an elegant work from Rosenkranz et al [32], measuring acetylsalicylic acid in plasma found significant lower ASA concentration than that achieved by us. [13,32] This apparent discrepancy may be explained by several reasons including the measured performed in Rosenkranz´s work was performed after only a single dose of ASA while in our work it was after four days of ASA treatment. Moreover, both acetylsalicylic acid and salicylic acid binds to albumin. Therefore, plasma determinations may result in detection of lower ASA levels than in serum. In addition, plasma half-life of acetyl salicylic acid is only 20 min and the fall in serum ASA concentrations is associated with rapid rise of serum salicylic levels. [33]

Incubation of Meg-01 cells with salicylic acid, also generated PLPs with higher content of Bak and Bax proteins. However, higher concentration of salicylic acid than ASA was needed to observe this effect. As speculation, it may suggest that the acetyl group of ASA could facilitate and contribute in some way to at megakaryocyte level sensitivity the expression of these pro-apoptotic molecules during PLPs formation. Future specific studies are needed to analyze more in depth this observation.

On the light of the present results, apparently the presence of ASA in Meg-01 cells promotes newly formed platelets with pro-apoptotic features. However, it could be also possible that these apoptotic alterations occur within mature platelets in the blood circulation. The experiments also suggested that ASA increased the content of Bak protein in Meg-01 cells by themselves. Therefore, Bak protein from Meg-01 cells could later be inherit by the newly formed PLPs. Apoptosis is a mechanism linked to platelet generation from megakaryocytes. Interestingly, the presence of ASA enhanced the number of PLPs generated from PMA-stimulated Meg-01 cells. However, although mature platelets have diminished their capability to synthetize new proteins with the present experimental design we cannot discard the possibility that ASA may also increase the content of apoptotic proteins. Specific future studies are then needed to clarify this issue.

We must point out that to accelerate PLPs production Meg-01 cells were stimulated with the protein kinase C (PKC) agonist PMA. It is well established that PMA works as accelerator of PLPs production by Meg-01 cells [34]. However, it is true that PKC exist in different isoforms and due to the multifold actions of PKC isoforms the molecular mechanisms by which PMA induces differentiation of Meg-01 cells to initiate platelets production probably are unspecific and with high complexities. In this regard, since it has been reported pro- and anti-apoptotic effects on PKC in various cell types, we cannot discard that PMA stimulation of Meg-01 cells could in some way influence the apoptotic characteristics of the newly formed PLPs. However, it is important to remark that, independently of the presence of ASA, Meg-01 cells was always stimulated with PMA. Several studies have also reported antagonistic effects of ASA on PKC signaling better than enhancement [35] but in the present experiments, the presence of ASA increased PLPs production by PMA-stimulated Meg-01 cells.

Platelets generally contain only 5–8 mitochondria per cell [36]. However, metabolically, platelets are quite active. For example, compared to resting mammalian muscle cells, platelets have much higher levels of ATP-turnover essential to regulate the pro-thrombotic activity of platelets [36,37]. The present study, supports that PLPs derived from Meg-01 cells contain mitochondria, and their density was not significantly modified when they were derived from ASA-incubated and ASA-non incubated Meg-01 cells. It was suggested by the fact that citrate synthase activity, an enzyme used as marker of mitochondria density [31,38], was similar between them. Interestingly, citrate synthase activity in PLPs was similar to that reported in human circulating platelets [39,40]. Moreover, the presence of mitochondria in PLPs was also supported by the detection of the cytochrome C oxidase activity, enzyme involved in the mitochondrial respiratory chain.

Mitochondria has a central participation in apoptosis (for a review, see ref. [41]). Indeed, the intrinsic apoptotic pathway is also called the mitochondrial pathway owing to the essential involvement of mitochondria in apoptosis [42,43]. Several studies have shown that mitochondria are major source of ROS, and ROS are main inductors of platelet apoptosis [44,45]. However, in the experiments the content of mitochondrial gp91-phox NADPH subunit, that promotes mitochondrial superoxide anion production, was reduced in the PLPs derived from ASA-incubated Meg-01 cells while the content of the mitochondrial manganese-superoxide dismutase isoform, (SOD2), was increased as compared with those PLPs from Meg-01 cells incubated without ASA. These observations may initially suggest that mitochondrial ROS generation may not be main cause of the higher apoptotic sensitivity of the newly formed PLPs from ASA-incubated Meg-01 cells.

One of the used biomarker to determine apoptosis was $\Delta\Psi m$. In this regard, during apoptosis, $\Delta\Psi m$ decreases as a process associated with the opening of the mitochondrial permeability pores and loss of electrochemical gradient. Mitochondrial depolarization was detected by the reduction of red to green fluorescence ratio using the JC-10 dye. It happened to a greater extent in PLPs generated from ASA-incubated Meg-01 cells considering the variation of $\Delta\Psi m$ between basal and A23187-stimulated conditions. It was noteworthy that at basal condition, the ratio red to green fluorescence was significantly higher in the newly PLPs generated from ASA-incubated Meg-01 cells than in those from Meg-01 without ASA. It is difficult to determine the optimal value of $\Delta\Psi m$ in all type of cells and, in our knowledge, this is the first time that it was determined in PLPs.

Although, as mentioned, mitochondrial ROS could not be main cause of the higher apoptotic sensitivity of PLPs generated from ASA-incubated Meg-01 cells, it is important to keep in mind that at high $\Delta\Psi m$ the mitochondrial respiratory chain acts as producer of ROS [46]. Therefore, it could be also possible that an increased ROS production by the mitochondrial respiratory chain may has as defensive consequence the reduction of the expression of other ROS sources, i.e. gp91-phox NADH subunit, and/or increasing the expression of antioxidant defensive proteins such as SOD2. Future studies are needed to study these possibilities.

Platelet activation requires fully functional mitochondria [47]. In the experiments, PLPs from ASA-incubated Meg-01 cells shown a reduced activity of the complex IV component of the mitochondrial respiratory chain, cytochrome C oxidase, as compared with this enzymatic activity in PLPs-derived from non-ASA -incubated Meg-01 cells. This difference was only becoming apparent when PLPs were activated with calcium ionophore that

is when PLPs probably really need fully capacity to generate ATP. In this regard, Barile et al demonstrated that inhibition of platelet cytochrome C oxidase disrupts platelet function and even more inhibited platelet-mediated blood coagulation [48].

Control of mitochondrial quality is essential to maintain a functional mitochondrial population and it is regulated by mitophagy [49,50]. Therefore, mitophagy alterations may result in accumulation of a platelet population containing defective mitochondria.

Mitophagy is regulated through mitochondrial co-localization of PINK-1 and Parkin proteins [51]. In the experiments, PLPs generated from megakaryocytes incubated without ASA showed lower levels of mitochondrial PINK-1 protein than that in the mitochondria of PLPs-derived from ASA-incubated Meg-01 cells. In addition, although translocation of Parkin from cytosol to mitochondria is a PINK1-dependent phenomenon, mitochondrial content of Parkin protein was similar between both PLPs types. Although we could not discard that differences in the expression of mitophagy-related proteins may be occurring downstream to PINK-1 and Parkin proteins, lack of differences in mitochondrial recruitment of Parkin protein between the two PLPs types probably diminish the involvement of mitophagy alterations in the mitochondrial dysfunction seen in PLPs generated from ASA-incubated Meg-01cells. Accordingly, Walsh et al have reported that PINK1−/− mice platelets did not reveal changes in platelet function [52]. In addition, to control platelet mitochondrial functionality, other PINK-1 and Parkin-independent mechanisms have been suggested, including aldolase reductase and the p53 pathway [53].

It has been extensively demonstrated that NO binding to heme group of cytochrome C oxidase inhibiting its activity, which has been associated with apoptosis [54–56]. In our experiments, PLPs generated from ASA-incubated megakaryocytes showed higher NOS3 levels than those newly PLPs formed from Meg-01 cells without ASA. Moreover, the presence of the L-arginine antagonist, L-NAME, in A23187-stimulated PLPs generated from ASA-incubated Meg-01 cells, significantly reduced caspase-3 activity. Taken together, these results suggested that NO may at least partially be involved in the higher apoptotic sensitivity of PLPs generated from ASA-incubated Meg-01 cells. Future studies are warranted to study more to study more depth and detail the possible involvement of the NO-dependent pathway in the apoptotic ability of the newly PLPs generated from ASA-incubated megakaryocytes.

It is noteworthy that previous works have demonstrated that partial inhibition of electron transport by NO leads to backup of electrons in complexes I to III, and these more reduced forms of the complexes generate more ROS [57]. It then could be suggesting again that the increased SOD2 content and reduced gp91-phox NADPH oxidase could be defensive consequence of increased ROS production from the mitochondrial respiratory chain. Accordingly, it was reported that PINK1 accumulation identifies mitochondria undergoing oxidative stress [58].

Comments and Open Questions

We are aware that this is a descriptive study and that more studies are needed to elucidate the mechanisms involved in the promotor of the apoptotic characteristic seen in PLPs derived from ASA-incubated Meg-01 cells. In this regard, it is important to point out that apoptosis and platelet activation are two dissociated processes. As example, Gyulkhandayan et al [6] demonstrated in an elegant work that platelet activation is a quick and short-term platelet phenomenon while platelet apoptosis required much longer platelet stimulation time, which may be in accordance with the hypothesis that platelet apoptosis may be a mechanism to control thrombus growth.

One of the main points remaining to be analyzed is why the increased content of Bax and Bak proteins in PLPs from ASA-incubated Meg-01 cells did not result in activation of apoptotic-related pathways when they were at basal conditions. As speculation, it is probably that under unstimulated conditions apoptotic triggering mechanisms downstream to Bax and Bak proteins remain inhibited and solely after platelet stimulation were these mechanisms also activated. In this regard, it was reported that Bax protein is localized in the cytoplasm and it is redistributed to mitochondria in response to apoptotic stimuli, where induces cytochrome C release [59]. Moreover, it has been also demonstrated the involvement of PI3K-Akt pathway in the regulation of mitochondrial Bax translocation [59]. Further studies are warranted to analyze the regulation and involvement of all these signaling cascades in the apoptotic regulation of PLPs.

It is difficult to hypothesize the clinical consequences of the here in vitro observations. However, as speculation, platelets with a more prepared pro-apoptotic machinery could more easily enter in apoptosis under conditions of high platelet stimulation than platelets that did not show such pro-apoptotic preparation. In this regard, our findings could be in accordance with the clinical observation that chronic low ASA dose treatment was associated with minor incidence of myocardial infarction than unstable angina during an acute coronary syndrome [60,61].

In summary, the presence of low ASA concentration in megakaryocytes during PLPs generation promotes the generation of different PLPs in terms of apoptotic sensitivity, NOS3 expression and mitochondrial functionality. The better understanding of mechanisms modified by low ASA concentration may contribute to better known the anti-platelet effects of ASA which may also open new therapeutics targets and strategies to improve platelet inhibition.

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Disclosure Of Interest

The authors declare that they have no conflict of interest

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bjh short report

Paradoxical effect of SARS-CoV-2 infection in patients with immune thrombocytopenia

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Summary

Thrombocytopenia has been identified as a common complication of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection in the general population. In an attempt to determine the impact of coronavirus disease 2019 (COVID-19) in patients with immune thrombocytopenia (ITP), a retrospective single-centre study was performed. Thrombocytosis was observed in patients with chronic ITP after SARS-CoV-2 infection, frequently needing treatment adjustment or even discontinuation of therapy. Relapses and newly diagnosed cases showed a fast response after initial treatment compared to ITP. Reduced immune activity due to lymphopenia during COVID-19 could explain this paradoxical effect, although further studies are needed.

Keywords: immune thrombocytopenia, COVID-19, SARS-CoV-2, thrombocytosis, lymphopenia.

Immune thrombocytopenia (ITP) is an acquired disorder characterised by peripheral destruction of platelets as a result of loss of primarily or secondarily induced immune tolerance.[1] As in many other autoimmune diseases, some viruses have been identified as a trigger for the immunological activation of ITP, leading to the diagnostic episode or to a relapse. Since the outbreak of the novel coronavirus severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) in December 2019, millions of people have been infected worldwide. Although we already know some of the complications of coronavirus disease 2019 (COVID-19) in the general population, such as coagulopathy or thrombocytopenia,[2] the behaviour of the virus in patients with specific pathologies needs further investigation.

In an attempt to determine the impact of COVID-19 pandemic in a tertiary hospital for ITP in Madrid, a retrospective single-centre study was performed. The objective of our study was to describe the clinical and analytical evolution of patients with ITP infected by SARS-CoV-2.

Patients and methods

We analysed 142 patients with ITP diagnoses followed in our centre, based in our database. Data were collected to establish a Register of patients with ITP and COVID-19. Patients with ITP and confirmed COVID-19 between 1 March and 30 April 2020 were enrolled. SARS-CoV-2 infection was diagnosed either by polymerase chain reaction (PCR) on nasal-and-throat swab samples or serology. Serology was performed in patients with negative PCR but symptoms suggestive of COVID-19. The project was

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approved by the Ethics Committee of La Paz University Hospital.

Results

A total of 10 patients in our cohort were diagnosed with COVID-19, comprised of three women and seven men. The median (range) age was 75 (41–94) years. Eight patients had chronic ITP and two had newly diagnosed ITP associated with COVID-19. An ITP relapse was seen in two patients with chronic ITP, simultaneous to SARS-CoV-2 infection. Diagnostic evaluation showed that none of these patients had secondary ITP. Complete laboratory testing, including autoimmune study with anti-nuclear antibodies (ANAs), was done in all of them, with a negative result for the last variable.

During the study period, all patients with ITP diagnoses in our hospital were contacted be telephone either by a haematologist or nurse, in order to evaluate their situation during the COVID-19 outbreak. Among them, 15 patients were followed by a telemedicine programme and nine patients performed on-site control visits. Both patients with newly diagnosed ITP were detected at the Emergency Department of our hospital. Six patients with chronic ITP communicated their SARS-CoV-2 infection in their outpatient follow-up, whereas the rest notified it by telemedicine.

Among the patients with chronic ITP, four were receiving treatment with thrombopoietin receptor agonists (TPO-RAs), whereas the other four had no treatment. Two of the patients receiving TPO-RAs were simultaneously taking corticosteroids. Recent ITP relapse before virus diagnosis was the reason for starting therapy with corticosteroids in one patient, who also received oseltamivir; whereas, refractory ITP and cortico-dependence was the reason in the other. The patients with newly diagnosed ITP were initially treated with corticosteroids and intravenous immunoglobulins (Igs).

COVID-19 was diagnosed in eight patients by positive PCR, whereas two were diagnosed retrospectively by positive IgG serology. Table I summarises the demographic and clinical characteristics of the patients and the clinical diagnosis of COVID-19. During the study period, Public Healthcare policy recommended COVID-19 testing only in patients with suggestive symptoms. SARS-CoV-2 infection was tested by PCR in six more patients in our cohort, with a negative result. Widespread testing was not available until the pressure on the health system ameliorated after the initial outbreak. Admission to the hospital was required for five patients, due to clinical or analytical criteria of severe disease. At COVID-19 diagnosis, half of the patients had a platelet count of $<100 \times 10^9$/l (2–86×10^9/l). Lymphopenia ($<1 \times 10^9$ lymphocytes/l) was observed in five patients. Successive blood count tests were performed for seven patients after diagnosis. Due to pandemic restrictions, telemedicine was used for patients with clinical stability, for whom blood test follow-ups could not be performed. Tests from the patients with retrospective COVID-19 diagnoses were also not available. Figure 1A shows platelet evolution during the first week after SARS-CoV-2 infection was confirmed. Thrombocytosis (platelet count of $>400 \times 10^9$/l) was present in five patients in the first week after diagnosis of COVID-19. All patients had a platelet count of $>100 \times 10^9$/l at day 7 of infection, with a downward trend in the following weeks. Thrombocytosis lasts from 1 to 6 weeks, depending on the patient. It should be noted that clinical and radiological pneumonia were only observed in those patients with thrombocytosis.

Among the patients with chronic ITP, treatment adjustment was needed in some because of thrombocytosis. Patient 1, who was not taking any ITP-specific treatment, received anti-platelet therapy with acetylsalicylic acid because of extreme thrombocytosis ($>700 \times 10^9$/l). Patient 2 had had a relapse 14 days before COVID-19 diagnosis, receiving treatment with corticosteroids, intravenous Igs, oseltamivir and TPO-RAs (romiplostim), with a good response. At the time of infection, this patient's platelet count was 86×10^9/l and he was receiving a tapering regimen of corticosteroids, romiplostim and oseltamivir. In the first 7 days, an increase in platelets was observed, reaching 575×10^9 platelets/l and allowing a temporary discontinuation of ITP treatment. As shown in Figure 1B, persistent thrombocytosis was detected during the following weeks, even though TPO-RA was not administered during weeks 5 and 6, and was administered at half-doses between weeks 7 and 9, with subsequent discontinuation. In patient 3, treatment with TPO-RAs was reduced by 25% 5 days before virus detection because of a platelet count of $>300 \times 10^9$/l, whereas the patient had maintained a stable platelet level of around 110×10^9/l during the previous 3 months. This increase could be directly related to initial COVID-19 infection.

Regarding patients with the lowest platelet count ($<30 \times 10^9$/l), patient 7 was not receiving treatment because, historically, he had $>30 \times 10^9$ platelets/l so he did not fulfil treatment indication. At COVID-19 diagnosis, he had a relapse of ITP. Patient 8 relapsed a month before SARS-CoV-2 infection, so she was receiving treatment with corticosteroids and romiplostim for that reason. Patient 9 came to hospital because of dyspnoea and cough, and was diagnosed with COVID-19. At admission, he had 219×10^9 platelets/l and no treatment for ITP, as he had always had a normal platelet count. Then, 24 h later, this count dropped to 2×10^9 platelets/l, so treatment with intravenous Igs was initiated. Patient 10 visited the Emergency Department due to haemorrhagic symptoms, so treatment with corticosteroids was started. After anamnesis, we were aware that she had had clinical manifestations suggestive of SARS-CoV-2 infection, so serology was tested, which was positive.

Both ITP relapses and newly diagnosed cases had a good response after initial treatment, faster than is typically observed in other newly diagnosed cases. All the patients exhibited a normalised platelet count in <7 days.

In patients with ITP relapse (platelet count of $<30 \times 10^9$/l) in the context of COVID-19, thrombocytosis was not

Table I. Clinical and demographic characteristics of patients with ITP and COVID-19.

Patient number	Age, years	Type of ITP	ITP treatment at the moment of COVID-19 diagnosis	Comorbidities	Fever	Odynophagia	Persistent cough	Dyspnoea	Anosmia	Dysgeusia	Myalgia/ arthralgia	PCR done?	PCR result	Serology test result	Inpatient management	COVID-19 treatment
1	83	Chronic	None	None	Yes	No	No	No	No	No	Yes	Yes	Positive		No	HCQ + azithromycin
2	56	Chronic	Corticosteroids + romiplostim + oseltamivir	HIV	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Positive		Yes	HCQ
3	79	Chronic	Eltrombopag	None	Yes	No	No	Yes	Yes	Yes	No	Yes	Positive		No	None
4	84	Chronic	Eltrombopag	Dyslipidaemia, atrial fibrillation	Yes	No	No	Yes	Yes	Yes	No	Yes	Positive		Yes	HCQ + azithromycin
5	67	Chronic	None	Dyslipidaemia, type 2 diabetes, sleep apnoea syndrome	No	No	No	No	No	No	Yes	Yes	Negative	Positive (IgM, IgG)		None
6	74	Chronic	None	Dyslipidaemia, asthma	Yes	No	Yes	Yes	Yes	Yes	No	Yes	Positive		Yes	HCQ + azithromycin + tocilizumab
7	94	Chronic	None	Hypertension, COPD	Yes	No	Yes	No	No	No	No	Yes	Positive		Yes	HCQ
8	76	Chronic	Corticosteroids + romiplostim	Type 2 diabetes, Hypertension	No	No	No	No	No	No	Yes	Yes	Positive		No	None
9	55	Newly diagnosed	None	Dyslipidaemia	Yes	No	Yes	Yes	No	No	Yes	Yes	Positive		Yes	HCQ
10	41	Newly diagnosed	Corticosteroids	None	Yes	No	Yes	Yes	No	Yes	Yes	No	—	Positive		None

COPD, chronic obstructive pulmonary disease; HCQ, hydroxychloroquine.

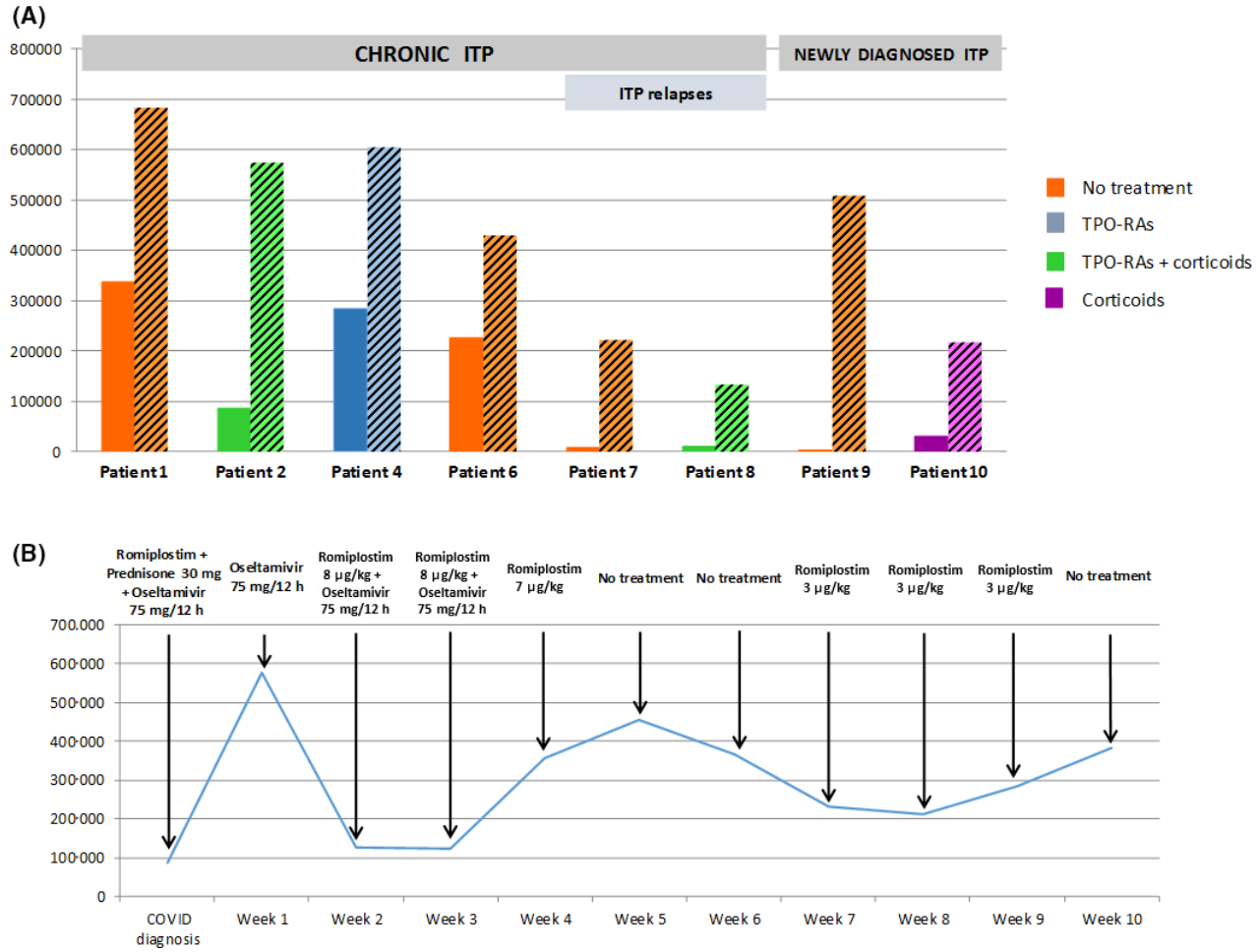

Fig 1. (A) Platelet count at day 1 (plain columns) and eight (striped columns) of the COVID-19 diagnosis is shown. All patients developed an evident increase in platelet count. Data from two patients could not be registered, as one patient was followed by telemedicine, while diagnosis of the other patient was retrospective. Patient 8 was also diagnosed with COVID-19 retrospectively, so the platelet count shown in the figure does not coincide with virus infection. (B) Platelet count and treatment adjustment of patient 2. [Colour figure can be viewed at wileyonlinelibrary.com]

reached. However, it is noteworthy that they had an important increase in their platelet count after SARS-CoV-2 infection. This increase was in comparison even higher than that observed in patients with chronic ITP and thrombocytosis.

Patients with newly diagnosed ITP associated with SARS-CoV-2 infection developed thrombocytosis in the first 10 days of treatment, starting an early tapering of corticosteroids and even reaching their suspension in <2 weeks. Anti-platelet therapy was started in patient 9 because of thrombocytosis.

Neither thrombotic nor haemorrhagic events appeared in this cohort, although one patient developed acro-ischaemic lesions in one finger, which could have been related to the endothelial dysfunction and arterial thrombotic disease induced by this infection. No mortality was observed during follow-up.

Discussion

Several studies have investigated the importance of platelet levels during SARS-CoV-2 infection and its prognostic relevance; however, none have reviewed this issue in patients with ITP. Thrombocytopenia, generally mild, has been a common finding among the general population with COVID-19, although the aetiological mechanisms are not well established.[3] Contrary to what has been recently published, we observed that patients with chronic ITP generally present early thrombocytosis after SARS-CoV-2 infection, frequently needing treatment reduction and even discontinuation of therapy. Relapses of chronic ITP in the context of COVID-19 also showed a fast recovery. It should be noted that corticosteroids tapering was started in the first 7 days of treatment in patients with newly diagnosed ITP associated with COVID-19. Consequently, treatment interruption was achieved in the first 10 days, earlier than we typically find in other idiopathic ITP, but similar to what has already been described in previous cases of ITP secondary to viral infections, typically observed in children.[4] Thrombocytosis has never been described in any recent SARS-CoV-2 pandemic publication, but it had already been observed in severe acute

respiratory syndrome (SARS) in 2003, when 49% of infected patients presented thrombocytosis following initial thrombocytopenia.[5] Increased TPO levels were detected in plasma of recovered patients with SARS, being postulated as a possible explanation for thrombocytosis.[6] Further studies are needed to evaluate platelet count evolution in patients with ITP and COVID-19.

Thrombocytopenia has also been well established as a risk factor for poor COVID-19 outcomes. The lower the platelet count, the higher the mortality becomes.[7–10] Consonant with these results, we did not observe any death due to COVID-19, given none of our patients developed persistent thrombocytopenia.

Many patients were treated with hydroxychloroquine. It has been reported that this drug may have an immunomodulatory effect on ANA+ patients that improves clinical manifestations of autoimmune diseases.[11] Nevertheless, the recovery of platelet counts in the patients with ITP in our present cohort cannot be a consequence of hydroxychloroquine treatment, as all our patients were ANA negative.

As observed in other viral infections, SARS-CoV-2 can act as a trigger for various autoimmune diseases. Although stronger evidence is required, some cases of ITP secondary to COVID-19 have already been published,[12,13] associating the inflammatory viral response with immunological tolerance dysfunction. Furthermore, the literature shows a probable correlation between COVID-19 and other autoimmune phenomena[14] based on increased auto-antibody production secondary to viral stimulus. In our present cohort, only two patients with chronic ITP relapsed in the context of viral infection and two newly diagnosed cases were detected. Nevertheless, various immune responses to SARS-CoV-2 have been studied. Lymphopenia is a common finding among infected patients, and is a prognostic factor that is directly related to mortality. Some studies have observed that both B and T lymphocytes decrease during active infection.[15,16] Given the physiopathology of ITP is based on platelet destruction by auto-reactive B cells and altered regulatory T lymphocytes, this decrease could promote a reduced platelet attack, leading to a low relapse rate. In addition, reduced immune activity could be the explanation for the observed thrombocytosis despite treatment discontinuation. Larger studies will be needed to support these associations.

In conclusion, although thrombocytopenia is a common finding in patients with COVID-19, and frequently associated with a poor outcome, we observed a different trend in patients with ITP. Thrombocytosis generally develops in these patients secondary to SARS-CoV-2 infection, although the aetiological explanation has not been established. Reduced immunological activity due to lymphopenia could explain this finding. In addition, relapse and newly diagnosed cases could be related to immunological dysfunction induced by the viral effect, as has been observed with other viruses. Given this is the first study of COVID-19 in patients with ITP, further studies are needed to clarify this premise.

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ORIGINAL ARTICLE

Clinical haemophilia

Registry of patients with congenital bleeding disorders and COVID-19 in Madrid

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Abstract

Introduction: We present the first registry of patients with congenital bleeding disorders and COVID-19. The study has been carried out in the Community of Madrid, which has the highest number of cases in Spain. The objective is to understand the incidence of COVID-19, the course of the disease if it occurs and the psychosocial and occupational impact on this population.

Methods: We included 345 patients (246 of haemophilia, 69 of von Willebrand Disease, two rare bleeding disorders and 28 carriers of haemophilia). A telephone survey was used to collect the data.

Results: Forty-two patients presented symptoms suggestive of infection by COVID-19, and in six cases, the disease was confirmed by RT-PCR. The cumulative incidence of our series was 1.73%. It is worth noting the complexity of the management of COVID-19 in two patients on prophylaxis with non-factor replacement therapy. Adherence to the prescribed treatment was maintained by 95.5% of patients. Although 94% were independent for daily living activities, 42.4% had a recognized disability and 58% required assistance, provided by the Madrid Haemophilia Association (Ashemadrid) in 75% of cases. Only 4.4% of consultations were held in person.

Conclusions: Patients with congenital bleeding disorders infected with SARS-CoV-2 presented a mild course of the disease that did not require admission. Their identification and treatment by a specialist team from a Haemophilia Treatment Center are essential to make a correct assessment of the risk of haemorrhage/thrombosis. COVID-19 had a major impact on the psychosocial aspects of these patients which must be remedied with recovery plans.

KEYWORDS

COVID-19, haemophilia A, rare bleeding disorders, telemedicine, von Willebrand disease

Maria T. Álvarez Román and Nora Butta Coll contributed equally to this work.

1 | INTRODUCTION

On March 11, the WHO declared the COVID-19 pandemic, with Spain, and specifically Community of Madrid (CM), being one of the areas most affected.[1,2] On February 25, the Hospital Universitario La Paz (HULP) treated the first patient diagnosed with COVID-19 in the CM. Since then, our hospital has housed a large number of SARS-CoV-2–infected patients. In this context, the Haemophilia Treatment Center (HTC) at HULP, which is a national benchmark for the management of patients with congenital bleeding disorders (CBDs), has established action protocols to try to minimize the risk of contagion. These protocols, which followed the recommendations established by the World Federation of Haemophilia (WFH), were designed so that the patient would come to hospital only in emergencies.[3] The management of these patients was made possible by the establishment of telemedicine (TM) programmes by the HTC.

Although patients with CBDs are not believed to be at an increased risk of developing COVID-19, and that if it does occur it need not be more severe than in the general population, there are no studies to confirm this.

The main objective of the study is therefore to find out the incidence of COVID-19 in this group of patients, and how the disease progresses if it occurs.

As secondary objectives, we seek to find out what support they have needed from the HTC and how it has been delivered, to identify where the infection has been managed, to analyse if there has been communication between the HTC and the Health Service where the patient was treated, to assess adherence to treatment, and to identify the factors influencing the potential loss of adherence. Moreover, we aimed to assess impact of lockdown on the musculoskeletal system and to collect psychosocial aspects of the strict lockdown such as the need of family and social support, employment situation and granted dependency/disability benefit.

Aspects of quality of care given by the HTC were also collected.

2 | MATERIAL AND METHODS

Following the recommendations of the WFH, on March 16 we (HTC) agreed a protocol recommending patients not to come to the centre except if they were bleeding. At that time, a TM programme was established through phone calls to find out the medical and psychosocial status and needs of the patient.

Data were collected to establish a registry of patients with CBDs and COVID-19.

The project was approved by the Ethics Committee of La Paz University Hospital.

The multidisciplinary team that cares for these patients (haematologists, rehabilitation physicians, pharmacists, nurses, clinical trials team and social workers) developed a home-made database with all the variables listed in Annex 1 (Appendix S1). Epidemiological, demographic and clinical data were collected to see the cumulative incidence, evolution and impact of the pandemic on this population.

Both the HTC and the Ashemadrid databases were used to identify patients.

The data collection process involved the haematologists, the clinical trials team and the social workers of Ashemadrid. The latter is a key element in identifying the needs of patients, since having advised them not to attend the HTC we had no record of SARS-CoV-2 infection, apart from patients who had required hospital admission or had attended the HULP emergency department.

Patient data were collected from 20 April to 4 May 2020.

It was explained to the patient that the data provided to us were collected to find out the incidence of COVID-19 in patients with CBDs and how their habits have changed due to the pandemic.

They were asked for informed consent and assured that all their data would be treated confidentially in accordance with European and Spanish Regulation.

Patients were divided by pathologies: severe haemophilia A/B(SHA/SHB), moderate haemophilia A/B (MoHA/MoHB), mild haemophilia A/B (MiHA/MiHB), von Willebrand's disease (vWD), carriers of haemophilia, and rare bleeding disorders (RBDs).

Patients presenting respiratory symptoms were classified as possible (mild acute respiratory infection for which no microbiological diagnostic test has been performed) or confirmed (it meets laboratory confirmation criteria by PCR) cases, according to the Ministry of Health's Procedure for Action against Cases of Infection with the New Coronavirus SARS-CoV-2.[4] Patients without dyspnoea, expectoration or haemoptysis were considered to have a mild respiratory infection, according to the technical document 'Home management of cases under investigation, probable or confirmed of COVID-19, Version of February 17, 2020', from Spanish Ministry of Health. A haematologist evaluated the symptoms reported by the patients to see if they met the possible case criteria defined by the Ministry of Health at the time in which the study was conducted. Those patients who did not comply with the possible case definition, but had symptoms highly suggestive of COVID-19 infection such as anosmia, were referred to HTC to have a PCR performed.

Psychosocial state of patients was evaluated with the 'home-made' questionnaire shown in Appendix S1 that, despite not being a validated tool, was adequate according to the criteria of the social workers involved in this study.

Qualitative data were expressed by absolute frequencies and percentages. Cumulative incidence was estimated with the 95% confidence interval. Statistical analyses were performed by Statistical Unit of La Paz University Hospital.

3 | RESULTS

3.1 | Patients' characteristics

The register included 345 patients of whom 246 were diagnosed with haemophilia, 69 with vWD, two with RBDs and 28 were haemophilia carriers. Patients included represent the whole population with bleeding disorders in CM because we are the only Center,

TABLE 1 Distribution by age and pathology

	Haemophilia A = 215					Haemophilia B = 31							
Age	Severe = 139	Moderate = 11	Mild = 44	With inhibitors = 21	Severe = 14	Moderate = 7	Mild = 5	With inhibitors = 5	vWD = 69	Carriers = 28	RBDs = 2	Total = 345
0-14	47	1	10	8	1	1	2	3	9	2	0	84
15-44	64	2	7	10	7	4	0	1	26	9	1	131
45-64	26	7	22	3	4	1	3	1	21	13	1	102
>65	2	1	5	0	2	1	0	0	13	4	0	28

Service and Reference Unit of the National Health System (CSUR) for treatment of these disorders in this community.

Of 246 patients diagnosed with haemophilia, 215 had haemophilia A and 31 had haemophilia B. The distribution according to severity was as follows: 159 patients with SHA (20 of them with inhibitor), 11 with MoHA and 45 with MiHA (one of them with inhibitor). As regards haemophilia B, 19 had SHB (five of them with inhibitor), seven MoHB and five MiHB. See Table 1 for distribution by age and pathology.

Thirty-six patients met possible case criteria, and six met COVID-19 confirmed case criteria. Table 2 shows features of suggestive COVID-19 and non-COVID-19 population.

Since the set of patients observed in the HULP sample was not independent of the aggregated data arranged by the CM, to compare cumulative incidences, we calculated the 95% confidence interval of the point estimate with the HULP data on May 4, when we contacted last patient. The cumulative incidence of our series is 1.73% with a 95% confidence interval (0.359; 3.110).

Of the 345 patients, 37 were HIV-positive. In this HIV/COVID-19 subgroup, there were two confirmed cases and three possible cases. With respect to the overall number of patients, the incidence of HIV and COVID-19 is 0.58% with the 95% confidence interval (0; 1.4) so the incidence of COVID-19 is not higher in patients with congenital blood disorders with HIV.

Despite the fact that our registry included a paediatric population and that other authors have described severe Kawasaki-like disease in that population, we did not find any cases. Neither did we observe the neurological symptoms associated with COVID-19 described in the literature such as stroke and Guillain-Barré syndrome. Even so, we will continue monitoring patients to detect the appearance of new symptoms.[5,6]

The clinical evolution of all patients who became infected with SARS-CoV-2 was mild, with none requiring specific treatment for infection.

The most commonly used HIV treatment was emtricitabine/rilpivirine/tenofovir alafenamide.

Thirty-one of the patients diagnosed with haemophilia were on prophylactic treatment with non-factor replacement therapy for haemophilia (NFRTs), emicizumab or concizumab, and one more on gene therapy. Two of them developed COVID-19 and deserve special mention. The first one diagnosed with SHA without inhibitor was in prophylaxis with emicizumab 6 mg/kg once a month. As comorbidities, he presented HIV (CDC category c3 and CD4 >200 cells/mm^3) and was receiving antiretroviral treatment with lamivudine and darunavir-cobicistat. The HTC contacted the patient by phone as part of their TM programme identifying that he had anosmia and a dry cough. At that point, he was sent to the HTC, where he tested positive using SARS-CoV-2 PCR. The chest computed tomography angiography did not show COVID-19-compatible affectation or pulmonary thromboembolism. He presented no elevation of inflammatory parameters (IL-6 or ferritin) and no activation of coagulation (no D-dimer elevation). Despite this, given the thrombotic risk that could be caused by COVID-19 together with the treatment with emicizumab we started treatment with low molecular weight heparin

TABLE 2 Features of suggestive COVID-19 and non-COVID-19 population

Variable	Population with suggestive COVID-19[a] n = 42 (%)	Population recognized as non-COVID-19 n = 303 (%)
VIH positive	11.9	10.56
Treatment with clotting factor concentrate	80.95	92.08
Treatment with non-factor replacement therapy	19.05	7.92
More than 65 y old	7.14	9.24
Close contact with people with symptoms	59.52	11.88
Lockdown accompanied	85.71	85.81
Contacted with healthcare system	64.29	4.29

[a]Suggestive included possible and confirmed cases.

(LMWH) at prophylactic doses with strict control with anti-Xa. The second patient, a male diagnosed with SHB with inhibitor, a healthcare worker, had discontinued treatment with concizumab 1 week earlier. He was SARS-CoV-2 PCR positive, and the only symptoms he presented were anosmia and dysgeusia with elevated D-dimer (twice its normal value) and lymphopaenia. He did not receive specific treatment for COVID-19 and fully recovered at 2 weeks. Neither had a severe case of COVID-19 or required hospitalization.

The 63 patients who presented some type of respiratory/infectious symptom in the last few months were evaluated by a haematologist who decided whether or not they were suggestive of COVID-19. Thirty-six contacted the health services, and only 20% contacted the HTC, performing the diagnostic tests in this population. The rest were managed in primary care or in hospitals closer to their homes, but without specialization in congenital blood disorders.

3.2 | Management of the disease from healthcare and community support system

Ninety-five per cent of patients can be considered adherents, respecting the dose and number of doses prescribed by the haematologist. The rest either spaced out the factor infusions out of fear of being left untreated for bleeding or of becoming infected when going to the hospital pharmacy, or self-adjusted the treatment to the lower physical activity. Those who had loss adhesion did not show an increase in the number of breakthrough bleeding.

With regard to physical activity, 57% had adjusted it to lockdown, and patients who requested it were sent a guideline of exercises to be done at home, prepared by the HTC rehabilitation team.

3.3 | Psychosocial aspects

When the psychosocial aspects were evaluated, most of them considered that they had been under a stricter lockdown than the rest of the population. In those who felt they had been under a stricter lockdown, the reasons were fear, concern and ignorance of how SARS-CoV-2 could affect them due to them having a chronic disease. Eighty-six per cent were spending their lockdown with their families. And while 94% were independent for activities of daily living, 42.4% had a recognized disability.

During lockdown, 58% of the patients had required assistance. This was provided 75% by Ashemadrid, 22.5% by the HTC and 2.5% by CM Social Services. Some of them (16.7%) acknowledged that their, or that of their household, employment situation had worsened.

The impact on quality of care was assessed with indicators such as the number of appointments postponed or cancelled. Of the appointments scheduled between March 9th and May 5th, 32.4% were cancelled, 50% were postponed, 13.2% were made by TM, and only 4.4% of the patients were seen in person. Of the 82% of appointments that were cancelled or delayed, 23% were at the request of the patient, who was afraid to come to the centre.

The majority of patients accepted the TM programme. In these months, a home delivery programme has been implemented with the objective of reducing the number of visits to hospitals minimizing the contagious.

4 | DISCUSSION

This is the first registry of patients with congenital blood disorders and COVID-19. Although there are initiatives from different pharmacovigilance organizations such as the *European Haemophilia Safety Surveillance (EUHASS)* to collect cases as they occur, no data are yet available.[7] To date, only one case of a patient with SHA and COVID-19 with good progression has been published.[8] One of the strengths of this registry is the large number of patients with a rare disease such as congenital blood disorder.

In our series, we observed a relative higher cumulative incidence of this disease with few complications than the rest of the CM

population. This may be due to the close monitoring of the patients that took place during this period of time, both by the HTC and by Ashemadrid, which has allowed us to identify more cases, which initially had not come to the HTC, and to carry out more tests than in the rest of the CM. On the contrary, a cumulative incidence similar to that of all population has been reported for patients with rheumatic diseases[9] that had had a close follow-up. Nevertheless, this observation was not considered conclusive because most of the patients had adopted a very strict preventive strategy against COVID-19 since the beginning of the epidemic.[10]

When we analysed the subgroup of patients with HIV-infected CBDs, we found no difference in the cumulative incidence of COVID-19 with respect to patients with HIV-negative CBDs. This may be because haemophilia and HIV patients in our series have good control of their HIV infection (CD4 counts above 200/mm^3 and undetectable viral load) and good adherence to ART (antiretroviral therapy), so their risk of contracting COVID-19 is no greater than that of HIV-negative subjects.

Another finding of our study is that patients with blood disorders do not develop a more serious disease than the rest of the population, as none of them required admission or specific treatment for COVID-19.

On the other hand, a large number of patients with congenital blood disorders who developed COVID-19 were managed in the Outpatient Care setting without establishing any communication with the HTC. Special attention should be paid to this fact, as the management of these patients by non-expert blood disorder staff can lead to erroneous decisions. Haemostatic treatment may be inadequately modified or the interactions it may cause in coagulation tests may be misinterpreted, which may lead to a poor assessment of the blood disorder produced by SARS-CoV-2 and an incorrect assessment of the haemorrhagic/thrombotic risk of patients with CBDs and COVID-19. On our registry, the two patients with severe haemophilia treated with NFRTs who developed COVID-19 were identified by telephone and referred to the centre for evaluation in the hospital setting. One of them received prophylaxis with LMWH, which shows the complexity and the difficult handling that this type of patient can have and the need for it to be provided in specialist centres. Thanks to the early identification of both patients through TM, it was possible to design the most appropriate therapeutic approach for each case.

Adherence to their usual treatment was maintained by most of the patients, who respected the dose and number of infusions prescribed by the haematologist. Active monitoring of our patients revealed that 10 of them had spaced out their factor infusions for fear of being left untreated in the event of bleeding, or infection on their way to the hospital pharmacy, or they had adjusted their treatment due to the reduced physical activity resulting from lockdown.

Another of the strengths of this work has been the assessment of the impact in the social and employment contexts. The key to this was the networking of Ashemadrid's social workers and the professionals of the HULP's HTC. This coordinated work allowed for a more comprehensive understanding of the determinants of patient and family health during the pandemic.

The evaluation of variations in the social and employment situation will enable Ashemadrid to prioritize the design of aid plans for submission to the authorities, with the aim of improving and directing its interventions towards the recovery and normalization of the state of well-being that these patients had prior to the pandemic.

There is no doubt that in Madrid, one of the places most affected by the pandemic where all the resources available for patients with COVID-19 have been used, the quality of health care for patients with CBDs has been adversely affected. Rehabilitation programmes, screening, training programmes for factor home management, inclusion in clinical trials, and access to more innovative treatments were discontinued, and there was a significant delay or even cancellation in the performance of diagnostic tests, elective surgeries, etc.

The health authorities such as the Ministry of Health or the Community of Madrid, as well as different scientific societies such as the Spanish Society for Quality Care, are working on a plan to restore health care that takes account of both patients affected by COVID-19 and other patients with other pathologies in which numerous healthcare actions, some of which are of vital importance, have been discontinued and which may have had a very negative impact on the population.[11]

Likewise, the recovery of the new normality is not going to be fast, so it is especially necessary to implement TM programmes. These programmes, which have long been used in haemophilia, are now essential for proper management of patients with CBDs during the pandemic.[12-15]

5 | CONCLUSION

The TM programme established at the HTC and the intense work carried out by Ashemadrid to contact patients have led to the preparation of this registry. Early detection of patients is essential for appropriate management by professionals with experience in congenital blood disorders, since interpretation of laboratory tests can be complicated in some groups of patients such as those being treated with emicizumab, and assessment of haemorrhagic/thrombotic risk in patients with CBDs with COVID-19 is difficult.

The coordinated work between the Patients' Associations, whose function is to identify the medical and psychosocial needs of patients, and the HTCs with their multidisciplinary teams is fundamental in the design of plans that try to minimize the impact of the pandemic on a vulnerable population such as patients with congenital blood disorders.

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Drs. Álvarez-Román and Jiménez Yuste have participated as speakers, in advisory boards and sponsored symposia with Novo Nordisk,

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AUTHOR CONTRIBUTIONS

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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Haemophilia WILEY

LETTER TO THE EDITOR

COVID-19 and telemedicine in haemophilia in a patient with severe haemophilia A and orthopaedic surgery

We describe the case of a patient with severe haemophilia A who underwent major orthopaedic surgery managed postoperatively by telemedicine (TM). The case is a splendid example of the implementation of TM and good collaboration between a Comprehensive Hemophilia Treatment Center (CHTC) and a local hospital.

This is a 56-year-old patient diagnosed with severe haemophilia A without an inhibitor. The patient's medical background includes HBV infection, HCV (treated and cured) and HIV positive under treatment with highly active antiretroviral therapy (HAART). Due to severe, painful and disabling bilateral knee arthropathy, the patient underwent surgery for a left total knee arthroplasty (TKA) in January 2013, followed by a right TKA in 2015. The left TKA had become painful at the last annual check-up, and X-rays showed severe loosening of the prosthetic components. The patient also had a pronounced arthropathy in the elbows and ankles. The patient was being treated with rFVIII tertiary prophylaxis at 40 IU/kg every 48 hours. In January 2020, the first stage of a two-stage revision arthroplasty of the left TKA was performed with suspected infection (not confirmed) by implanting an articulated spacer. On 9 March, he was admitted for the second stage of revision to the left knee. Although the situation was becoming more complex in our hospital due to the spread of COVID-19, we decided to perform the surgery given that the patient had already travelled to the hospital. On 10 March, a long-stem tumoral prosthesis was implanted, and this was because resection of the distal femur was required due to severe osteolysis secondary to prosthetic loosening (Figure 1A). A bolus of rFVIII was administered preoperatively at a dose of 50 IU/kg, followed by continuous infusion. Preinfusion FVIII levels were 1.8%; no inhibitor was detected with a Cmax 181.9% after 20 minutes. Four hours after surgery, the patient presented an FVIII level of 113.5%. Twenty-four hours after surgery, having assessed the risk/benefit of admission, it was decided to discharge the patient from hospital for safety reasons, due to the high risk of infection with SARS-CoV-2. At that moment, given the progression of the pandemic, the Orthopaedic Surgery Department wards would be handed over to care for COVID-19 patients, at that point 80% occupied by such patients. The patient's blood test from that morning presented Hb 12.7 g/dL and a FVIII level of 92.3%. Physical examination: Surgical wound of good aspect, without signs of bleeding, range of movement on sagittal plane (ROM): complete extension and 30° flexion, quadriceps force 3-/5 (according to MRC muscle strength scale), transfers of weight using supports. Postoperative X-ray showed that the prosthesis had been correctly implanted.

Given the impossibility of maintaining continuous infusion during the transfer to his centre, the patient was switched to 40 IU/kg bolus infusions every 8 hours for 2 days until contacting the regional hospital in his home town to adjust treatment depending on FVIII levels, continuing treatment as per Table 1. The patient was transferred by ambulance to his home town, 400 km away from our hospital. On the same day, we contacted the patient upon his arrival to assess bleeding, pain experienced during the trip and to record constants such as BP and temperature. These were checked three times a day for the first 10 days. The patient reported that he had had no bleeding during the journey home and that the pain had been well controlled with metamizole.

Upon discharge of the patient, the Rehabilitation Department set out its guidelines for analgesic and physical therapy to encourage early recovery of the knee. This included assisted mobilizations, progressive muscle strengthening of the quadriceps, proprioception exercises and re-education of gait using technical aids. After 4-6 weeks, it was recommended that he start working on stairs and ramp exercises.

During the early postoperative phase, haematologists, physiatrists and orthopaedic surgeons participated in daily videoconferences to communicate with the patient. Physiatrist assessed the progress in joint mobility and haematologists the presence of bleeding. During the late postoperative period, the patient was assessed by emailing the images of his knee and the haematologists adjusted the treatment in coordination with the local haematologist.

The only complications were a subcutaneous haematoma on the lateral side of the left thigh on day + 6; we assessed this using photographs sent by the patient (Figure 1B), with full resolution three days later, bleeding from one of the sutures during a dressing change (day + 14) and an isolated *Cutibacterium acnes* in periprosthetic skin tissue, which was considered to be contamination, as the patient had no signs of local or systemic infection.

The patient was in daily email contact with our Hematology Department, which in turn kept in close contact with the regional hospital haematologist to adjust treatment as necessary. Samples of blood to track FVIII dosage were taken at the patient's home, and he was later informed of the new treatment guideline by our Hemophilia Unit. Physiotherapy sessions were conducted remotely online with the help of the patient's relatives. On day + 26, the patient was pain free. He had reached 90° flexion, showed good motor control and was walking 2000 steps a day with the help of two canes. Now, the patient is fully recovered by walking 300 metres a day in his home

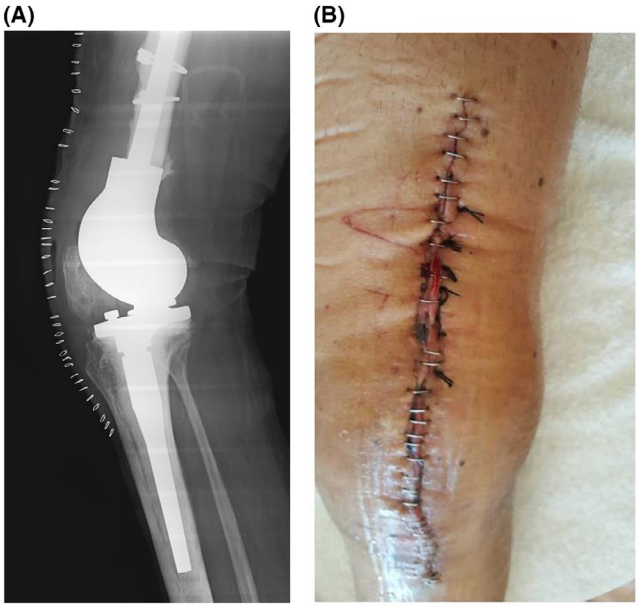

FIGURE 1. Figure 1, A Lateral radiograph of the long-stem tumoral prosthesis implanted in the left knee due to severe loosening of the primary prosthesis. B, Left knee on day+3.

TABLE 1 Haematological postoperative protocol used in this patient

Days postsurgery	FVIII levels	Dose guidelines
+1	92.3%	40 IU/kg/8 h
+3	FVIIIb: 60% Cmax FVIII: 170%	40 IU/kg/12 h
+6	FVIIIb: 89% Cmax FVIII:159%	25 IU/kg/12 h
+9	FVIIIb: 55% Cmax FVIII:111%	25 IU/kg/12 h
+13	FVIIIb: 43% Cmax FVIII:110%	37 IU/kg/24 h
+16	FVIIIb: 21% Cmax FVIII:139%	37 IU/kg/48 h

Note: FVIIIb: level FVIII before infusion, Cmax FVIII 20 min after infusion

environment due to the current nationwide lockdown. He will soon start exercising with stairs and obstacles. The patient has already returned to working from home.

Telemedicine (TM), defined as the provision of healthcare services remotely, is an expanding discipline,[1] although it is less commonly used in Spanish state-run hospitals than we would hope. This is mainly due to legal barriers that seek to protect confidentiality and patient data; economic barriers, given the high costs of implementation; and sometimes psychological barriers both for the patient and for the health professionals who tend to prefer face-to-face visits.

Since June 2019, our hospital has been working on the implementation of a TM model in the Haemostasis Unit, currently focusing on the most prevalent pathologies such as ITP or venous thromboembolic disease.

Our hospital is one of the largest healthcare centres in the Spanish public network and is therefore one of the hospitals that have treated the most patients with COVID-19 during the last two months, with an average of 900 patients hospitalized per day.

Early on in the health crisis, we designed a protocol to facilitate TM programmes for patients with congenital coagulopathies. The objective was to keep patients, many of whom were immunocompromised, such as the case in this study, away from the hospital to minimize the risk of infection from SARS-CoV-2.

Great progress in TM, such as videoconferencing, audiovisual link and remote patient-monitoring tools, has always been achieved in times of crisis. Its application in the hospital routine will allow patients to address healthcare issues quickly and offers medical staff a continuous flow of real-time patient's health data.[2,3] So, the healthcare situation caused by COVID-19 has accelerated the rapid implementation of these services, even for the management of this disease.[4] In this sense, we consider telemedicine to be a good strategy for monitoring patients who, following the recommendations of the World Federation of Haemophilia, should not attend hospital.[5]

The above case is a good example of interaction between a CHTC formed by a team of professionals (orthopaedic surgeons, physiotherapists, haematologists and nurses) specializing in the management of patients with congenital coagulopathies and a regional hospital with professionals who attend patients with very diverse pathologies.

There is not much experience in literature regarding the use of TM for haemophilia patients, but the little evidence that there is highlights that it is very useful for patients who live a long distance from the CHTC as in the case with the patient described.[6-9] However, the global pandemic situation we are experiencing is likely to change the attitude towards remote consultations, both for patients and for professionals alike.

According to Valentino et al, in the context of a pandemic, TM has the potential to increase convenience and facilitates access of patients to medical specialists, improves patient safety and allows better budget management, limiting exposures to patients while reducing the burden on healthcare facilities allowing them to deal with the sickest patients[10]

In the case presented, it should be noted that telemedicine was useful both in case of the Hematology Service and the Orthopedic and Rehabilitation Departments.

In conclusion, we have presented the case of a 56-year-old patient diagnosed with severe haemophilia A that underwent a revision total knee arthroplasty and provided with postoperative follow-up via TM. The patient was managed by a multidisciplinary team of haematologists, rehabilitation physicians and orthopaedic surgeon and is a magnificent example of the use of TM and good collaboration between a CHTC and a district hospital, at the time of the COVID-19 pandemic. The first case of COVID-19 was detected in Spain on 20 February 2020. From that moment on, the number of diagnosed cases increased rapidly, with Spain, and Madrid in particular, being

one of the most affected areas in Europe. Our CHTC is a national benchmark for the management of these patients. From the very beginning of the health crisis, our hospital housed a large number of COVID-19 patients, forcing us to set up TM programmes to manage haemophilia patients safely.

The main disadvantage of TM in this case was the inability to conduct a direct physical examination. But we have learned that in this kind of cases in which surgery cannot be delayed, follow-up can be performed by TM. If the situation of the pandemic continues, the intervention would be performed in regional hospitals guided by our orthopaedic surgeons through TM.

KEYWORDS

COVID-19, haemophilia A, orthopaedic, surgery, telemedice

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AUTHOR CONTRIBUTIONS

MTAR and VJY designed the work. MTAR wrote the letter. HdlC, ECRM, MMS and MIRP were responsible of patient's surgery, treatment and follow-up. NVB, SGB, PA, TC, EG and EMM were responsible of data management. All authors analysed, revised critically and gave final approval of the manuscript.

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bjh research paper

Platelet and immune characteristics of immune thrombocytopaenia patients non-responsive to therapy reveal severe immune dysregulation

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Summary

Multifactorial mechanisms leading to diminished platelet counts in immune thrombocytopaenia (ITP) might condition the ability of patients with ITP to respond to treatments. Examining their platelet and immune features, we aimed to detect singular characteristics of patients with ITP who do not respond to any treatment. We studied patients with chronic primary ITP who had been without treatment, or untreated (UT-ITP), for at least six months; included were responders to agonists of thrombopoietin receptors (TPO-RA), patients who showed no response to first- and second-line treatments (NR-ITP), and healthy controls. Platelets from NR-ITP patients exposed a reduced amount of sialic acid residues. Increased loss of platelet surface sialic acid residues was associated with increased platelet apoptosis. NR-ITP patients had an increased fraction of naive lymphocyte (L) B cells and a reduced LTreg (Lymphocyte T-regulator) subset. They also presented an anomalous monocyte and NK (Natural Killer) cells distribution. TPO-RA-treated patients seemed to recover an immune homeostasis similar to healthy controls. In conclusion, our results indicate a severe deregulation of the immune system of NR-ITP. The inverse correlation between loss of sialic acid and LTreg count suggests a potential relationship between glycan composition on the platelet surface and immune response, positing terminal sugar moieties of the glycan chains as aetiopathogenic agents in ITP.

Keywords: immune thrombocytopaenia, platelets activation, sialic acid, platelet apoptosis, lymphocyte T-regulator.

Immune thrombocytopaenia (ITP) is a rare disease (ORPHA 3002, OMIM 188030) of autoimmune origin, characterised by low platelet counts (below 100 000 platelets per microlitre) and diagnosed by exclusion of other causes of thrombocytopaenia. The initial event leading to antiplatelet autoimmunity remains unclear (Cooper & Ghanima, 2019), but there is strong evidence of the involvement of autoantibodies and of a deterioration of the regulatory compartment (Treg and Breg) and a polarisation of the immune response towards Th1 and Th17 (Ogawara *et al.*, 2003; Zufferey *et al.*, 2017). Moreover, platelets have non-haemostatic immune functions through the release of cytokines which may also play an important role in ITP (Kapur *et al.*, 2015; Kapur & Semple, 2016).

According to the surface expression of CD14 (lipopolysaccharide receptor) and of CD16 (low affinity Fcγ receptor III), monocytes can be categorised as classic ($CD16^-$, with higher phagocytic activity) or as non-classic monocytes ($CD16^+$, higher producers of tumour necrosis factor [TNF]-α). Some authors have revealed an anomalous distribution of monocytes in patients with ITP (Zhong *et al.*, 2012).

Natural killer (NK) cells and natural killer T lymphocyte (NKT) cells play a key role in connecting innate and adaptive immunity. NK cells participate in IgG alloantibody production in recipients of platelet transfusions (Sayeh *et al.*, 2004) and are also associated with human autoimmune diseases. NK cells can be classified, depending on the level of CD16 and CD56 expression, as $CD16^+CD56^{bright}$ or $CD16^+CD56^{dim}$. The role and phenotype of NK and NKT cells in the autoimmune processes of ITP are still unclear (Johansson *et al.*, 2004; Gutcher & Becher, 2007; Xu *et al.*, 2014).

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Antiplatelet antibodies facilitate platelet phagocytosis by spleen macrophages. However, the absence of antibodies in many of these patients suggests the participation of other mechanisms in the disappearance of platelets from the circulation. In this regard, several laboratories, including ours, have observed that an exacerbated platelet apoptosis and loss of sialic acid contribute to the establishment of thrombocytopaenia in ITP patients (Li *et al.*, 2015; Goette *et al.*, 2016; Justo Sanz *et al.*, 2019). Moreover, it has been reported that circulating cytotoxic $CD8^+$ T cells present in ITP patients are mediators of platelet destruction; the ability of these cells to directly lyse platelets was also confirmed *in vitro* (Olsson *et al.*, 2003; Zufferey *et al.*, 2017).

Bleeding symptoms in patients with ITP vary widely (Neunert *et al.*, 2015), as does their response to treatments. In some cases, patients do not respond to any of the available therapeutic approaches. These disparities may rely on individual platelet features and on immune compromise due to the different degree of involvement of mechanisms implicated in the ethiopathogenesis of this disease. On this basis we decided to study many primary variables related to either the platelet characteristics (platelet ability to be activated and to release the granule's content, apoptosis and their surface glycoside residues), or to the immune system (distribution of LB cells, LT cells, monocytes and NK cells, and cytokines profile in plasma), in order to find singular characteristics of patients with ITP who do not respond to any treatment.

Methods

Study design and participants

This was an observational, prospective and transversal study approved by The La Paz University Hospital Ethics Committee. Adult patients (older than 18 years) with chronic primary ITP were included (Rodeghiero *et al.*, 2009) after receiving signed informed consent from the patient.

Due to the rare condition of ITP, sample size was calculated according to the number of ITP patients attending the Haematology Unit of the University Hospital La Paz during the period 2015–2018. To be included in one of the three groups of the study, their clinical histories were studied to identify whether they fulfill the inclusion criteria. The first group consisted of untreated (UT-ITP) patients who did not need treatment for at least six months. This group included 28 UT-ITP patients [67·8% women, median age (25th–75th percentiles) 50 (32–71) years] who went into remission after a period of thrombocytopaenia lasting more than 12 months. The second group was formed by responders to agonists of thrombopoietin receptors [TPO-RA, patients who reached a platelet count $>30 \times 10^9$/l, with at least a two-fold increase in the baseline count and an absence of bleeding (Rodeghiero *et al.*, 2009)]. This group included 36 TPO-RA patients [55·6% women, 61 (41–79) years, 53·3% with eltrombopag, 46·7% with romiplostim]. All the patients treated with TPO-RA had previously been treated with corticosteroids and intravenous immunoglobulin (IVIG), and five of them had undergone a splenectomy. The third group was formed by 14 non-responders to first- and second-line treatments [NR-ITP, 64·3% women, 63 (51–82) years] (Audia *et al.*, 2017), whose previous treatments are shown in Table SI. Each case sample (ITP sample) was processed and studied simultaneously with a sample from one (two when possible) healthy control (sex and age matched) from the blood donor section of the Haematology Unit of La Paz University Hospital. So, a healthy control group of 104 individuals [55% women, 66 (38–79) years] was also included in the study.

The study excluded patients with uncontrolled hypertension, hyperlipidaemia, peripheral or coronary artery diseases, abnormal hepatic or renal function, those undergoing therapy with platelet-active drugs and those who had undergone a transfusion within 15 days of the study.

Preparation of platelet-rich plasma and washed platelets

Platelet-rich plasma (PRP) and washed platelets were obtained by whole centrifugation, as described in Data S1.

Determination of platelet surface receptors and platelet activation markers

Platelet surface expression of fibrinogen and von Willebrand factor, and fibrinogen receptors as well as platelet activation markers, were determined by flow cytometry as described in Data S1.

Measurement of caspase activity

Active caspase-3, −7, −8 or −9 were determined by flow cytometry in PRP, diluted with HEPES-buffer with 2 mmol/l Ca^{2+} and 2 mmol/l Gly-Pro-Arg-Pro (Sigma-Aldrich, Madrid, Spain) to prevent fibrin formation using CaspaTag kits (Millipore, Madrid, Spain).

Determination of platelet surface glycan exposure

Platelet surface glycan exposure was analysed by determining the binding of Alexa fluor 488-wheat germ agglutinin lectin (WGA, Invitrogen, Madrid, Spain) and FITC-*Ricinus communis* agglutinin (RCA; Vector Labs, Peterborough, UK), 1 μg/ml each. WGA binds to sialic acid and N-acetylglucosaminyl residues, and RCA is a galactose-specific legume lectin.

Isolation and analyses of peripheral blood mononuclear cells (PBMCs)

Blood collected in Cellular Preparation Tubes (CPTs) with sodium heparin (BD Biosciences, Madrid, Spain) was submitted to density gradient centrifugation (1800 ***g*** for 22 min at 23°C).

For detection of peripheral blood mononuclear cells (PBMC) subsets, specific antibodies were used, shown in Data S1.

Determination of cytokines

Cytokines shown in Data S1 were measured in serum samples with MILLIPLEX MAP Human Cytokine/Chemokine Magnetic Bead Panel (HCYTOMAG-60K, Merck, Madrid, Spain).

Statistics

Experimental data was analysed using GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA). The Shapiro–Wilk test was used to test the normal distribution of the population. Comparisons among groups of treatment were analysed with the Kruskal–Wallis test with a Benjamini–Hockberg correction to control the experimentwise error rate (ERR), considering a sciencewise false discovery rate (FDR) of 5% (software MEV 2.0; The Perl Foundation, Holland, MI, USA). Whenever this test result was significant, a *post hoc* Dunn's test of multiple comparisons was performed.

Results

Study population

As shown in Fig 1A, platelet counts for the ITP groups were lower than in the healthy controls. Moreover, the NR-ITP platelet count was also significantly lower than in the UT-ITP and TPO-RA-treated patients. Platelet count correlated with plasma levels of sCD40L ($\rho = 0{\cdot}304$, $P = 0{\cdot}014$) and IP-10 ($\rho = -0{\cdot}427$, $P < 0{\cdot}001$) (Figure S1).

Activation of platelets

Platelets from all patients with ITP showed an impaired activation of their fibrinogen receptor (Fig 1B). This deficiency was even more evident in the NR-ITP group. To determine

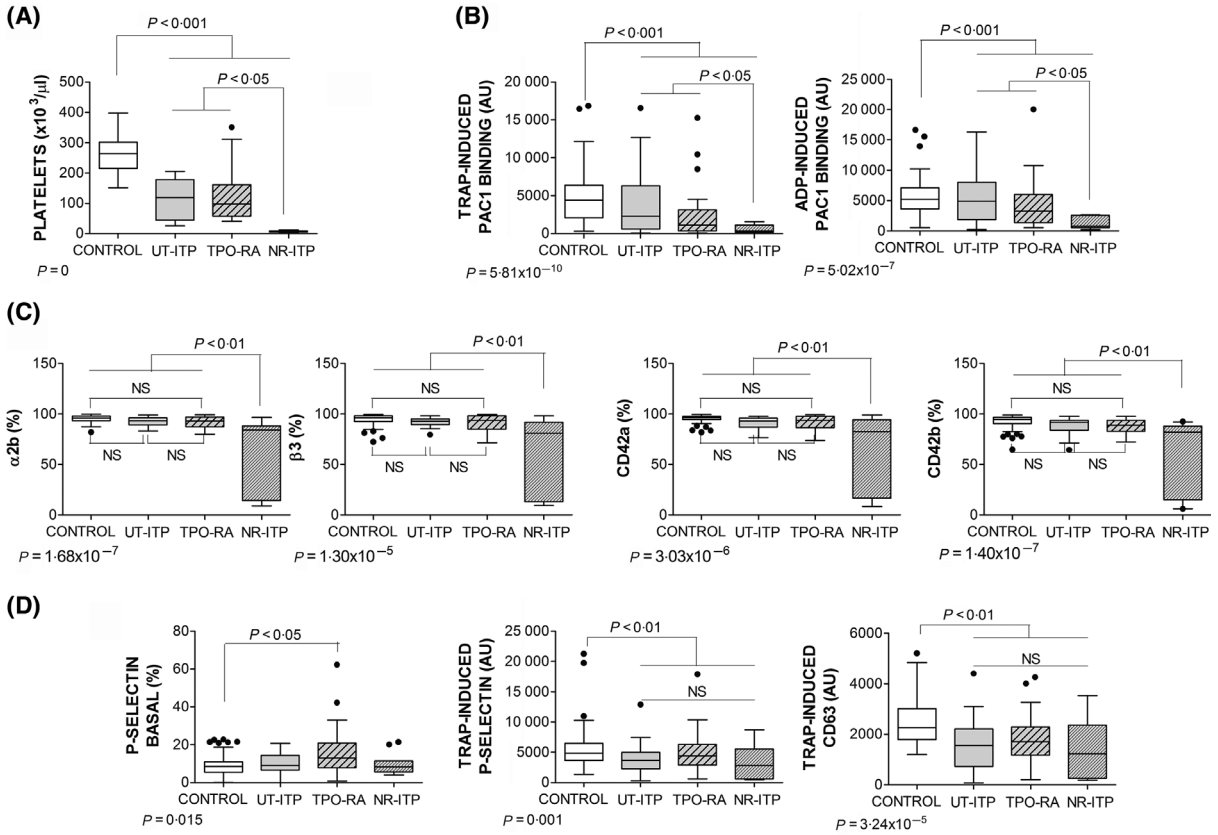

Fig 1. Platelet features in patients with ITP. (A) Platelet count in the four study groups: healthy individuals (control), untreated patients with ITP (UT-ITP), under treatment with agonists for the thrombopoietin receptor (TPO-RA) and non-responders to any treatment (NR-ITP). (B) Platelets stimulated with either 100 µmol/l TRAP or 10 µmol/l ADP were incubated with FITC-PAC1. (C) Quiescent platelets were incubated with PE-mAb-anti-αIIb or FITC-mAbs against β3, CD42a or CD42b. (D) Platelets in basal condition or TRAP-stimulated were incubated FITC-anti-P-selectin mAb and FITC-anti-CD63 mAb. Data were analysed by a Kruskal–Wallis test with a Benjamini–Hockberg correction considering a FDR of 5%. The adjusted *p* value is shown below each figure. When this test showed a significant result ($P < 0{\cdot}05$), a Dunn's test of multiple comparisons was performed and results are depicted within the figure. NS: no significant differences.

whether diminished binding of PAC1 was due to a reduction in the number of fibrinogen receptors, surface expression of their subunits was determined by flow cytometry. As shown in Fig 1C, only platelets from the NR-ITP group expressed fewer fibrinogen receptors. Moreover, NR-ITP patients also had fewer CD42a and CD42b receptors on their platelet's surface, indicating reduced von Willebrand receptor content.

Surface exposure of P-selectin and CD63 after stimulation with TRAP was also reduced in ITP patients (Fig 1D). Nevertheless, patients with ITP who had been treated with TPO-RA showed an increased basal exposure of P-selectin (Fig 1D).

Apoptosis in platelets

Platelets from TPO-RA-treated and NR-ITP patients had increased caspase-3, −7, −8 and −9 activity (Fig 2A). These increments appeared to involve a reduced ability of their platelets to be activated (Fig 2B).

Surface exposure of glycans in platelets

Loss of sialic acid from a platelet's glycoproteins can contribute to platelet disappearance through Ashwell–Morell receptors. We therefore tested galactose exposure as an indirect measurement of the loss of sialic acid through the binding of RCA lectin. Platelets from NR-ITP patients exposed more galactose than platelets from healthy controls and from the other ITP groups (Fig 3A). Moreover, NR-ITP also exposed more N-acetylglucosaminyl residues than the other groups (Fig 3A).

Binding of WGA and RCA correlated with caspase activities (Fig 3B) but did not appear to be related to the platelets' ability to be stimulated (data not shown).

Lymphocyte distribution

The fraction of lymphocytes in whole blood is shown in Table I. NR-ITP patients had a higher proportion of B lymphocyte (LB) cells compared with other ITP groups and with healthy controls. This increase could be due to a significant rise in the fraction of naive LB cells (Table I).

TPO-RA-treated ITP patients had a higher proportion of LBreg cells and of LTreg cells (Fig 4A), whereas UT-ITP and NR-ITP had a diminution in this last LT subset. It is of interest to note that loss of sialic acid from platelet surface glycans inversely correlated to the Tregs count but seems to have no relationship with the Bregs count (Fig 4B).

Monocyte and natural killer cell distribution

The patients with ITP had more monocytes than the healthy controls; however, their distribution varied depending on the ITP group. The fraction of classical monocytes was increased and the fraction of non-classical monocytes was decreased in the UT-ITP and NR-ITP groups (Fig 5A). On the other hand, the

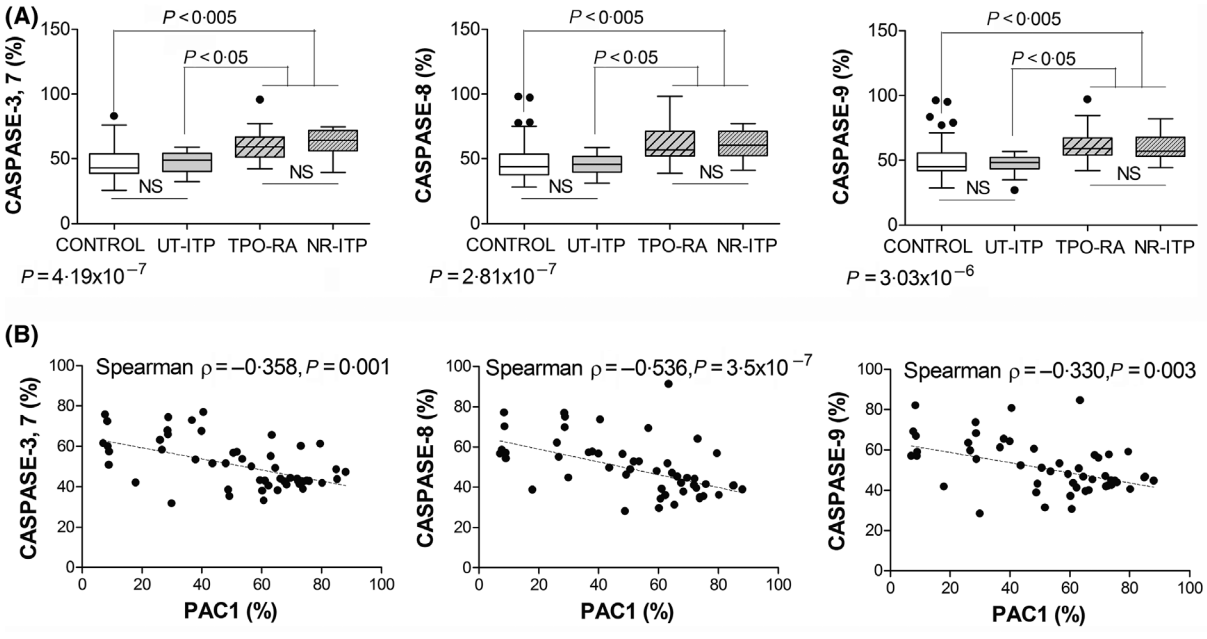

Fig 2. Caspase activity (A) was determined in quiescent platelets from controls and patients with ITP. Data are expressed as a percentage of positive cells. Data were analysed by a Kruskal–Wallis test with a Benjamini–Hockberg correction, considering a FDR of 5%. The adjusted *p*-value is shown below each figure. When this test was significant ($P < 0{\cdot}05$), a Dunn's test of multiple comparisons was performed and results are depicted within the figure. (B) Correlation between caspase activity and the platelet's ability to be stimulated through the TRAP-induced binding of PAC1 was determined by Spearman's test, and $P < 0{\cdot}05$ was considered significant.

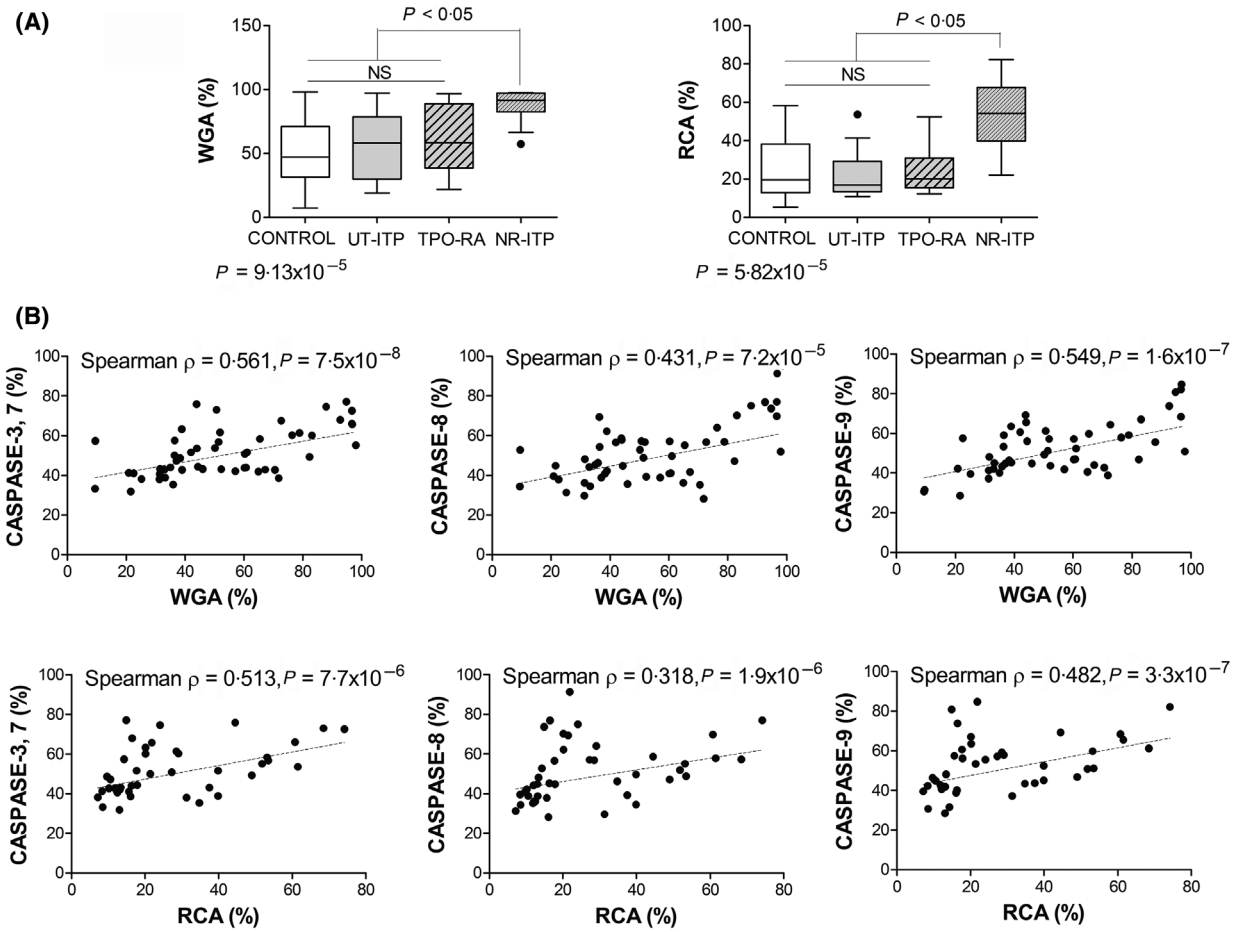

Fig 3. Lectin binding (A) was determined in quiescent platelets from controls and patients with ITP. Data are expressed as a percentage of positive cells. Data were analysed by a Kruskal–Wallis test with a Benjamini–Hockberg correction, considering a FDR of 5%. The adjusted *p* value is shown below each figure. When this test was significant ($P < 0{\cdot}05$), a Dunn's test of multiple comparisons was performed and results are depicted within the figure. NS: no significant differences. (B) Correlation between caspase activity and lectin binding was determined by Spearman's test, and $P < 0{\cdot}05$ was considered significant.

Table I. Lymphocytes in the different groups expressed as median (25^{th}–75^{th} percentile).

	Control	UT-ITP	TPO-RA	NR-ITP	*P*
Lymphocytes (%)	83·6 (77·3–86·0)	81·7 (74·2–85·9)	80·0 (70·7–85·5)	79·0 (47·9–85·8)	0·400
LT (%)	61·5 (55·8–67·5)	55·0 (52·0–62·2)	55·7 (44·3–65·1)	55·8 (44·3–64·4)	0·115
LT $CD4^+$ (%)	58·7 (52·5–66·1)	58·2 (43·1–61·5)	57·5 (49·5–67·3)	47·4 (23·9–65·4)	0·384
LT $CD8^+$ (%)	27·6 (23·3–33·4)	34·1 (24·6–44·3)	27·1 (22·0–38·3)	25·8 (20·6–32·4)	0·224
LT $CD4^+CD8^+$ (%)	0·4 (0·3–0·7)	0·5 (0·3–0·7)	0·5 (0·2–0·9)	0·5 (0·3–0·8)	0·785
LT $CD4^-$ $CD8^-$ (%)	4·7 (3·5–6·9)	5·8 (3·6–9·2)	4·6 (3·2–6·4)	4·0 (2·3–9·4)	0·580
LB%	11·2 (7·8–13)	9·8 (7·0–12·2)	9·7 (6·3–13·9)	13·7 (12·9–15·7)*	0·010
LB naïve (%)	68·2 (59·4–75·1)	71·7 (56·4–82·0)	76·8 (68·9–80·5)	84·1 (70·0–85·8)†	0·010
LB memory (%)	27·2 (18·0–34·8)	23·2 (11·9–42·1)	17·8 (8·5–24·4)	9·2 (7·7–56)	0·043
LB transitional (%)	4·9 (2·6–12·6)	2·1 (1·4–6·6)	9·2 (5·6—14·2)	6·2 (1·9–12·0)	0·063
LB plasmablast (%)	0·5 (0·3–0·9)	0·5 (0·3–0·8)	0·4 (0·3–0·6)	0·3 (0·2–1·2)	0·384

Data were analysed by a Kruskal–Wallis test with a Benjamini–Hockberg correction, considering a FDR of 5%. The adjusted *p*-value is shown in the column on the right. When this test was significant ($P < 0{\cdot}05$), a Dunn's test of multiple comparisons was performed.

*$P < 0{\cdot}01$ NR-ITP *versus* control, UT-ITP and TPO-RA groups;

†$P < 0{\cdot}05$ NR-ITP *versus* control.

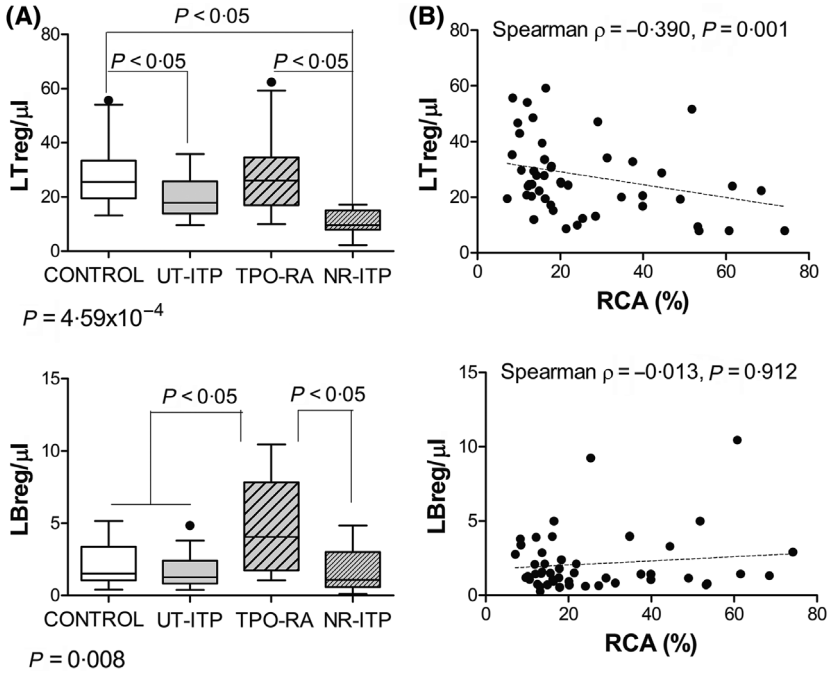

Fig 4. LTreg and Breg counts were determined by flow cytometry. (A) Data were analysed by a Kruskal–Wallis test with a Benjamini–Hockberg correction, considering a FDR of 5%. The adjusted *p*-value is shown below each figure. When this test was significant ($P < 0{\cdot}05$), a Dunn's test of multiple comparisons was performed and results are depicted within the figure. (B) Correlation between LTreg and Breg counts with RCA binding was determined by a Spearman correlation test. $P < 0{\cdot}05$ was considered significant.

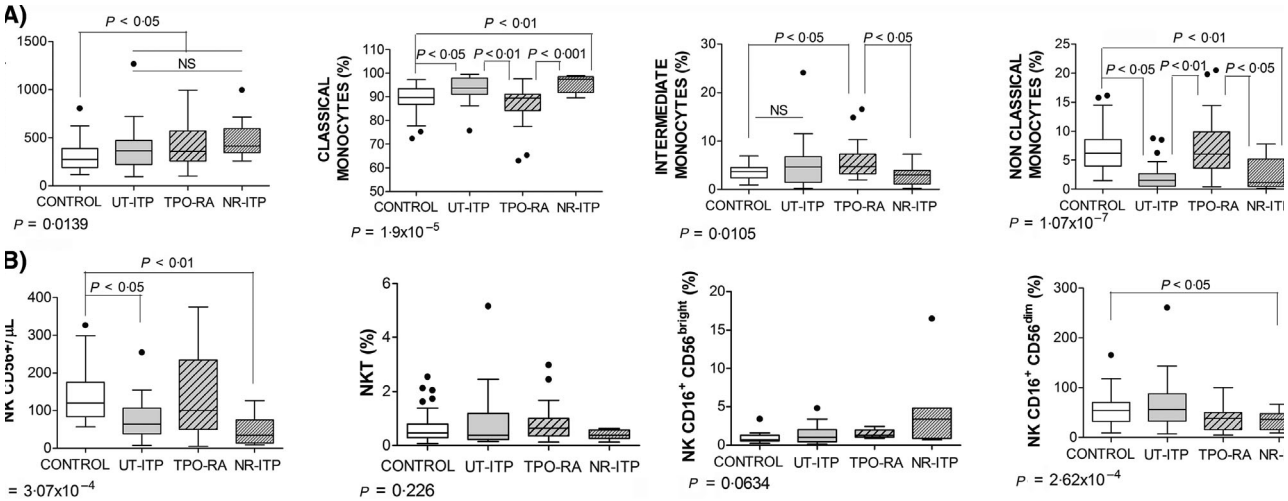

Fig 5. Monocyte count and percentage of monocyte subsets (A) and NK cell count and percentage of NK cell subsets (B) were determined by flow cytometry. Data were analysed by a Kruskal–Wallis test with a Benjamini-Hockberg correction, considering a FDR of 5%. The adjusted *p*-value is shown below each figure. When this test was significant ($P < 0{\cdot}05$), a Dunn's test of multiple comparisons was performed and results are depicted within the figure. NS: no significant differences.

TPO-RA-treated patients seemed to recover a monocyte distribution similar to healthy controls (Fig 5A). No differences were observed in intermediate monocyte subsets among groups.

UT-ITP and NR-ITP had low NK cell counts. NR-ITP patients presented a diminished NK $CD16^+$ $CD56^{dim}$ fraction (Fig 5B).

The proportion of NKT cells ($CD3^+$ $CD16^+$ $CD56^+$) was similar in all groups (Fig 5B).

Plasma level of cytokines in patients with immune thrombocytopaenia

Table II shows the plasma concentrations of the measured cytokines. The TPO-RA-treated patients had higher TNF-α levels than the other groups, whereas the NR-ITP group showed a reduced sCD40L concentration.

Table II. Level of cytokines in plasma expressed as median (25th–75th percentile).

Cytokine (pg/ml)	CONTROL	UT-ITP	TPO-RA	NR-ITP	*P*
IFN-	35·8 (19·6–93·0)	24·5 (11·6–63·8)	12·2 (3·5–28·9)	20·6 (5·8–146·0)	0·069
IL-10	0·19 (0·13–0·40)	0·29 (0·09–0·45)	0·27 (0·19–0·61)	0·30 (0·29–1·80)	0·304
sCD40L	19·6 (15·6–24·0)	15·2 (4·1–17·5)	15·9 (9·4–20·6)	1·3 (0·7–2·1)*	0·009
IL-17α	17·7 (10·9–49·6)	14·4 (3·1–38·6)	9·7 (1·9–38·6)	10·6 (3·1–69·0)	0·504
IL-1α	1·4 (0·2–11·5)	1·8 (0·5–75·9)	0·4 (0·1–2·3)	1·2 (0·6–27·2)	0·155
IL-1β	1·2 (0·7–1·9)	0·9 (0·8–2·7)	1·0 (0·6–3·1)	0·8 (0·7–2·2)	0·898
IL-2	1·3 (0·8–4·8)	1·1 (0·8–1·5)	0·9 (0·4–2·3)	0·9 (0·7–6·5)	0·188
IL-3	0·4 (0·2–0·5)	0·4 (0·1–0·6)	0·3 (0·1–0·4)	0·5 (0·3–0·7)	0·138
IL-4	1·2 (0·1–2·0)	0·2 (0·1–1·3)	0·2 (0·1–0·8)	0·2 (0·1–1·1)	0·231
IL-5	1·2 (0·6–5·6)	0·9 (0·6-1·4)	0·7 (0·6–2·1)	0·9 (0·5–7·3)	0·483
IL-6	2·6 (0·3–14·1)	2·1 (0·6–10·9)	1·8 (0·4–21·6)	2·1 (3·1–27·7)	0·203
IP-10	213·5 (173·0–241·3)	301·5 (236·1–470·0)†	296·0 (251·5–500·0)†	271·1 (176·3–466·3)†	0·000
MCP-1	598·0 (454·0–713·1)	520 (356·5–748·5)	534·0 (340·4–769·0)	378·0 (209·9–455·0)*	0·015
MIP-1α	12·1 (3·9–34·0)	13·7 (3·7–30·6)	15·6 (9·9–27·8)	23·6 (3·3–40·0)	0·988
TNF-α	10·3 (7·3–16·4)	14·5 (10·7–22·8)	27·5 (14·8–34·0)‡	14·4 (5·2–27·0)	0·001
TNF-β	0·03 (0·01–0·28)	0·03 (0·01–2·80)	0·02 (0·01–0·40)	0·06 (0·01–0·22)	0·234

Data were analysed by a Kruskal–Wallis test with a Benjamini–Hockberg correction, considering a FDR of 5%. The adjusted *P*-value is shown in the column on the right. When this test was significant ($P < 0·05$), a Dunn's test of multiple comparisons was performed.

*$P < 0·05$ NR-ITP *versus* control;

†$P < 0·05$ NR-ITP, UT-ITP and TPO-RA groups *versus* control;

‡$P < 0·001$ TPO-RA *versus* control.

All ITP groups had increased levels of IP-10.

Discussion

NR-ITP demonstrated increased apoptosis (caspase activities) compared to controls and UT-ITP. In addition, NR-ITP had the highest loss of platelet sialic acids compared to controls, UT-ITP and TPO-RA.

Caspase-mediated apoptosis is one of the mechanisms to eliminate platelets from circulation (Kile, 2014). Platelets from patients with ITP showed significantly higher phosphatidylserine surface exposure (Justo Sanz *et al.*, 2019), and in the TPO-RA and NR-ITP groups, enhanced caspase-3, −7, −8 and −9 activation (present results). Increased apoptosis has also been reported in other two ITP cohorts, one consisting of ITP patients who had been receiving different therapies and the other consisting of patients who had suspended treatments two weeks prior to sample collection (Goette *et al.*, 2016; Deng *et al.*, 2017).

Platelets' caspase activities inversely correlated with their ability to be stimulated. A similar relationship between these variables was observed in platelets from patients with myelodysplastic syndromes (Martin *et al.*, 2013). In addition, the lowest ability of platelets of NR-ITP patients to be activated might also be related to their decreased exposure of fibrinogen and von Willebrand factor receptors (Fig 1C). This observation suggests the impairment of a mechanism shared by both receptors, i.e. their synthesis or transport to platelet surface.

In accordance with other authors (Revilla *et al.*, 2019), we also observed that platelets from NR-ITP patients had lower levels of sialic acid in their surface glycans. Clearance of desialylated platelets through the hepatic Ashwell–Morell receptors has been proposed as an alternative mechanism for platelet disappearance from circulation (Li *et al.*, 2015). Nevertheless, despite the platelet count being lower and the loss of sialic acid being higher for NR-ITP *versus* TPO-RA group (Figs 1A and 3A), there was no difference in caspase activities between both groups (Fig 2A). Grodzielski (Grodzielski *et al.*, 2019) tested ITP plasma samples on platelets from healthy controls and observed that only 35% induced both desialysation and apoptosis, while others induced either apoptosis (25%) or desialylation (20%), or did not affect the platelets' features (20%). Taken together, these observations indicate that apoptosis and loss of sialic acid are not necessarily related.

Platelets from NR-ITP patients bound more WGA than other groups, indicating that their platelets exposed more N-acetylglucosaminyl residues. This fact indicates that the glycome composition of these platelets had more differences with platelets from healthy controls and from other ITP groups than the loss of sialic acid. An increment in exposure of N-acetyl glucosamine residues may induce platelet clearance from circulation through the integrin αMβ2 (Mac-1) receptor, present on the surface of macrophages, as reported for cooled platelets which also have an augmented N-acetyl glucosamine exposition (Li *et al.*, 2016).

Present and previous results from our group indicate the enhanced exposition of P-selectin in quiescent platelets from TPO-RA-treated patients (Justo Sanz *et al.*, 2019). Other groups have described higher plasma-soluble P-selectin levels and have proposed that its contribution increases the risk of

suffering thrombotic events (Garabet *et al.*, 2017; Frelinger *et al.*, 2018). Diminished release of P-selectin and dense granule content after agonist stimulation of platelets from patients with ITP might be due to their desensitisation to applied stimuli (Frelinger *et al.*, 2018).

It has been reported that there was a correlation between refractoriness to intravenous immunoglobulin or steroids and the presence of anti-GPIb-IX antibodies (Zeng *et al.*, 2012; Peng *et al.*, 2014) targeting the N-terminal ligand-binding domain (LBD) of GPIbα (Quach *et al.*, 2018). These mAbs (monoclonal antibodies) can directly activate GPIb-IX, leading to an intracellular signalling that produces P-selectin exposure and loss of sialic acid (Yan *et al.*, 2015; Hoffmeister & Falet, 2016). We also observed a significant reduction in the platelets' sialic acid in the NR-ITP patients; however, we failed to observe an increased exposure of P-selectin in this group. Since we did not evaluate antiplatelet antibody profiles in our cohort we do not know whether this discrepancy might be explained because our patients did not have anti-LBD of GPIbα mAbs. Another reason for this difference between results could be due to the fact that P-selectin exposure is shear-dependent (Quach *et al.*, 2018), and we performed our experiments under static conditions.

We also observed certain features that indicated an imbalance in immune cells distribution. In agreement with Ebbo *et al.* (2017), we noted that the number of $CD19^+$ B lymphocytes and $CD4^+$ and $CD8^+$ T lymphocytes were similar in UT-ITP and TPO-RA treated patients and healthy controls. Conversely, the LB percentage and LB naïve frequency in NR-ITP patients was higher compared with these groups, maybe because most of them were treated with rituximab, a B-cell depletion therapy. B cells which had re-expanded following rituximab showed important differences in their repertoire (phenotypic and functional characteristics). Reconstitution of $CD27^+$ memory B cells is often slow (from months to years) and this group had the highest frequency of LB naïve, probably due to this previous treatment.

The $CD3^-$ $CD56^+$ NK cells count was diminished in the UT-ITP and NR-ITP groups. El-Rashedi *et al.* (2017) also observed a significant reduction in the percentage of NK cells

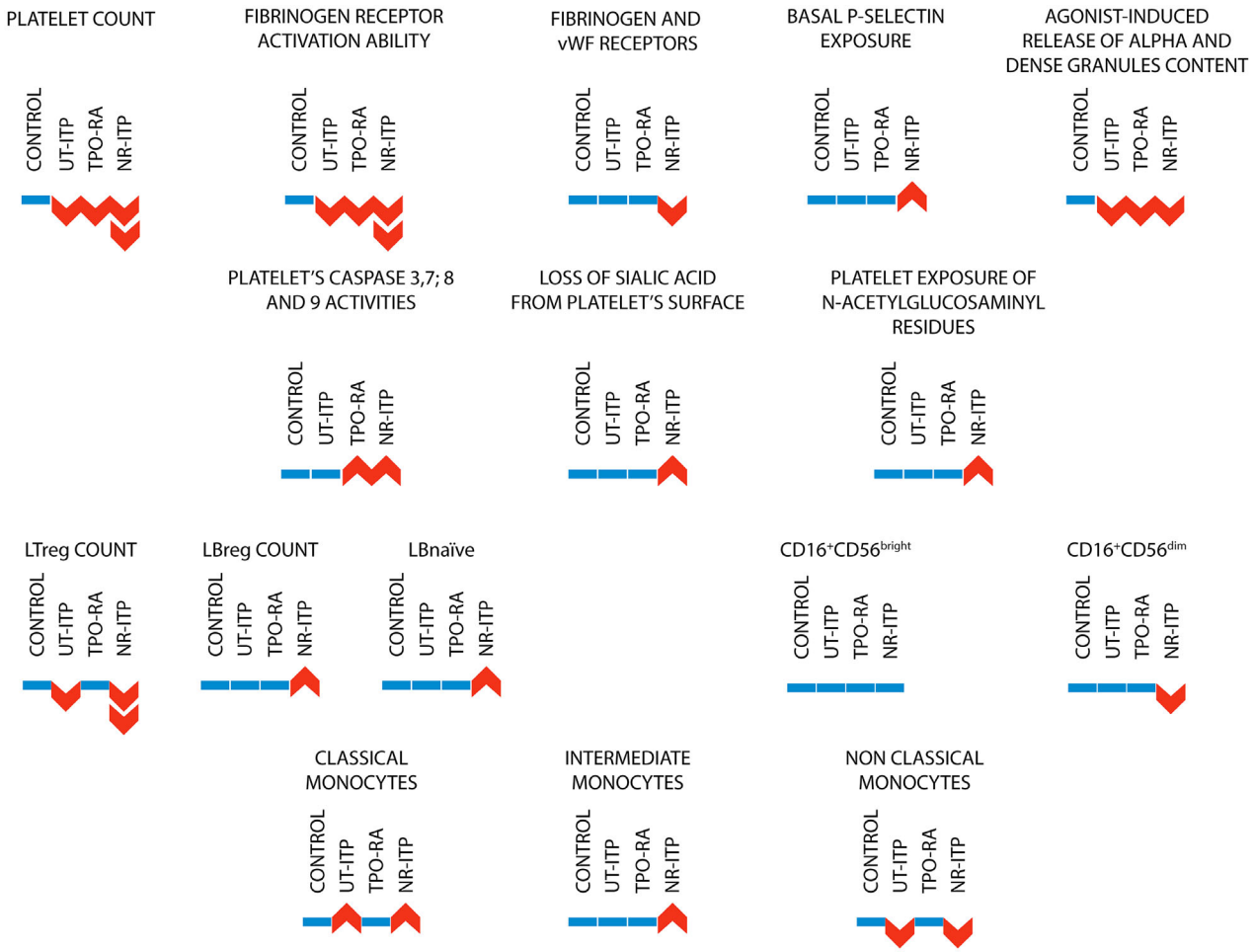

Fig 6. Schematic representation of platelets and immune cell characteristics of ITP patients, either untreated (UT-ITP), or responders to agonists of thrombopoietin receptors [TPO-RA), or non-responders to therapy. [Colour figure can be viewed at wileyonlinelibrary.com]

in patients with ITP compared with healthy controls, with a maximum reduction in the acute form of the disease. Conversely, Garcia-Suarez *et al.* (1993) reported increased $CD3^{-}$ $CD56^{+}$ NK cells in ITP patients, particularly in those refractory to therapeutic treatments. Nevertheless, Semple and Freedman (1994) demonstrated that patients with chronic ITP had functionally suppressed NK cell activity despite normal percentages and absolute counts of NK cells.

We observed a significant decrease in the $CD56^{dim}$ subset in NR-ITP patients. A similar reduction in the $CD56^{dim}$ fraction has been reported for young ITP patients at the onset of the acute disease and before receiving any steroid or immunosuppressive drug treatments (Talaat *et al.*, 2014; Zahran *et al.*, 2018). No differences were found in the NKT cell count among the groups.

All the patients with ITP had a higher absolute number of monocytes. Despite the non-classical monocyte population subset producing higher TNF-α after stimulation, TPO-RA-treated patients had an augmented plasma level of TNF-α, but a non-classical monocyte frequency similar to that observed in healthy controls. So, cytokine production does not seem to be linked to monocyte subpopulation frequency in ITP.

Zhong *et al.* (2012) have studied a cohort of patients with ITP, most of whom were being treated with TPO-RA and, in agreement with our results, did not find differences in the rate of the classical monocyte subset between the healthy controls and the TPO-RA-treated groups. On the contrary, they found an increase in the non-classical subset only in those patients with $<50 \times 10^9$ platelets/l, whereas we did not observe an increment in non-classical subset in the NR-ITP group, in which the platelet count was $11 \pm 3 \times 10^9$ platelets/l.

Patients with ITP reportedly show a cytokine imbalance towards IL-2 and IFN-γ as well as impaired Treg and Breg compartments (McKenzie *et al.*, 2013). The number and function of circulating and splenic Tregs and their frequency in the bone marrow of patients with ITP are reduced (McKenzie *et al.*, 2013; Audia *et al.*, 2017). Our results showed that TPO-RA treatment appeared to reverse Treg diminution, restoring a healthy distribution state. Similar results have been reported previously (Bao *et al.*, 2010). Moreover, TPO-RA administration in ITP was associated with the restoration of Fc-R balance towards the inhibitory FcγRIIb on monocytes (Liu *et al.*, 2016). Additionally, in a mouse model of ITP, treatment with a murine TPO-RA (AMP4; Amgen) normalised platelet count and significantly diminished serum antiplatelet IgG antibodies (Kapur *et al.*, 2019). So, TPO-RA appears to have a beneficial effect on immune response. Nevertheless, one of the limitations of our study was that patients were recruited only once the response to TPO-RA was achieved; therefore, a longitudinal study would provide more information regarding TPO-RA effects.

Patients with active ITP have been demonstrated to exhibit significantly higher Th1/Th2 ratios (McKenzie *et al.*, 2013). Nevertheless, we observed no differences between the Th1/Th2 cytokine profiles in plasma between the healthy controls and the patients with ITP, except for a decrease in IFN- in the TPO-RA treated group.

CD40L is stored in the platelets' granules and is exposed on the platelet membrane after stimulation; once exposed, CD40L is shed and sCD40L released (Menchen *et al.*, 2009). Some studies have reported increased plasma concentrations of sCD40L in patients with chronic ITP (Nagahama *et al.*, 2002); however, we found similar plasma concentrations in the healthy controls and in the UT-ITP and TPO-RA-treated patients. Gudbrandsdottir *et al.* (2017) have recently reported that in patients with chronic ITP, levels of sCD40L increased after six months of treatment with TPO-RA, but then decreased to pretreatment values that were similar to those found in healthy controls. We observed a significant reduction in sCD40L in plasma from NR-ITP patients. As platelets are considered the main source of sCD40L, this might be due to the low platelet count in this group of patients (Henn *et al.*, 2001). Regarding IP-10, we observed that the lowest platelet counts were accompanied by the highest levels of IP-10. In keeping with this result, it has been reported that IP-10, a cytokine involved in Th1 polarisation, is increased in plasma from active ITP patients (Gu *et al.*, 2010).

In conclusion, platelets from NR-ITP patients had more signs of apoptosis and a different composition of surface glycans, accompanied by a diminished LTreg population and a higher LB naïve percentage, indicating a severe deregulation of the immune system (Fig 6). Taken together, these observations suggest a potential relationship between glycan composition on the platelet surface and immune response, positing terminal sugar moieties of the glycan chains as aetiopathogenic agents in ITP. In support of this hypothesis, it has been reported that sialylated antigens regulate dendritic cells, which induce *de novo* Treg (regulatory T) cells when loaded with sialylated antigens, and inhibit the generation of new effector T cells as well as the function of existing ones (Perdicchio *et al.*, 2016). Knowledge of glycome composition might be useful to predict disease outcome and response to therapeutic treatments (in ITP patients).

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wrote the manuscript. All authors approved the manuscript. NVB, MTAR, IFB, MMS, MIRP, VJY are paid instructors at Novartis, Pfizer, Roche and NovoNordisk. VJY and MTAR are consultants at Roche, NovoNordisk, Pfizer, Takeda, SOBI and Bayer. NVB and MTAR are Consultants for Novartis.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Relationship between platelet count and plasma levels of sCD40L and IP-10. Correlation was determined by Spearman test and rho- and *p*-values are shown. $P < 0{\cdot}05$ was considered as significant.

Table SI. IVIG: intravenous immunoglobulin; $n = 14$ patients; 9 women (64·29%), 5 men (35·71 %). Median (25th–75th percentile) group age: 79 (51–82).

Data S1. Supplementary Files.

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Platelet Apoptosis and PAI-1 are Involved in the Pro-Coagulant State of Immune Thrombocytopaenia Patients Treated with Thrombopoietin Receptor Agonists

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Abstract

The treatment goal for patients with immune thrombocytopaenia (ITP) is to raise platelet counts to levels that minimize or stop bleeding. Thrombopoietin receptor agonists (TPO-RAs) have been successfully and extensively employed as second-line therapy for ITP. However, TPO-RAs have a small but significant increase in the risk of thrombosis. The aim of this study was to elucidate the mechanisms involved in the pro-coagulant effect of TPO-RAs to take them into account when considering their use in ITP patients with concomitant diseases/conditions that might increase risk of suffering thrombotic events. Eighty-two patients with chronic primary ITP (40 untreated and 42 undergoing TPO-RA therapy) and 112 healthy individuals were recruited. The patients with ITP undergoing TPO-RA therapy presented a pro-coagulant profile due to the formation of a more fibrinolysis-resistant clot because of increased plasminogen activator inhibitor-1 (PAI-1) levels. Increase in platelet content of PAI-1 might be the result of the effect of TPO-RA during megakaryopoiesis, as suggested by experiments performed in MEG-01 cells. Moreover, patients under TPO-RA treatment presented an enhanced pro-coagulant activity associated with microparticles and an increased platelet apoptosis that causes a higher exposure of phosphatidylserine and, consequently, a larger surface for the binding of the prothrombinase complex.

Keywords
- immune thrombocytopaenia
- platelet apoptosis
- PAI-1
- thrombopoietin receptor agonists
- platelet function

Introduction

Immune thrombocytopaenia (ITP) is an autoimmune disease characterized by a low platelet count ($\leq 100 \times 10^9$/L) due to platelet destruction and insufficient platelet production.[1] Patients with a similar platelet count can have different bleeding manifestations,[2] and some seldom bleed despite having a very low platelet count. This fact suggests that factors other than the platelet count are involved in maintaining haemostasis within a physiological range in patients with ITP. Supporting this, Frelinger et al reported that platelet function in ITP is consistent over time and is associated with bleeding severity.[3] Moreover, we and other authors have reported an increase in platelet-derived and red blood cell-derived microparticles (MPs) in patients with ITP,[4–6] both with a broad haemostatic activity. So, compensatory mechanisms to thrombocytopaenia seem to exist, and although they might appear somewhat protective, under certain circumstances, might be responsible for the slight but increased risk of thrombosis in patients with ITP.[7,8] Álvarez Román et al, in a study of haemostasis performed using rotational thromboelastometry (ROTEM), reported that patients with ITP showed a pro-coagulant profile. However, a wide range of clot formation profiles were observed.[6] Considering that thrombotic complications in patients with ITP could be due to the disease itself[9,10] or a consequence of ITP therapy,[11–13] the variations observed in the study by Álvarez Román et al[6] could rely on differences in therapy received by patients from that study's cohort.

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Among the drug therapies for ITP, thrombopoietin receptor agonists (TPO-RAs; romiplostim; Nplate; Amgen, Thousand Oaks, California, United States; and eltrombopag; Promacta, Revolade; Novartis, Basilea, Switzerland) are being successfully and extensively employed as second-line therapy. However, TPO-RAs have a small but significant risk of thrombosis.[14,15]

Considering that the use of TPO-RAs for treating ITP and other thrombocytopaenias is increasingly widespread,[16–18] we aimed to elucidate the mechanisms involved in the procoagulant effect of TPO-RAs to take them into account for achieving a global haemostasis as physiological as possible.

Methods

Subjects and Study Design

A prospective, observational and transversal study was conducted with 82 patients with chronic primary ITP, as defined by Rodeghiero et al,[1] and 112 healthy individuals recruited from the Blood Donor Section of the Hematology Unit of La Paz University Hospital.

The study excluded patients with uncontrolled hypertension, hyperlipidaemia, peripheral or coronary artery diseases, abnormal hepatic or renal function, those undergoing therapy with platelet-active drugs and those who had undergone a transfusion within 15 days of the study.

The La Paz University Hospital Ethics Committee approved the experimental protocol. The research study was conducted in compliance with the Helsinki Declaration and after receiving signed patient informed consent.

Preparation of Platelet-Rich Plasma and Washed Platelets

Human peripheral blood samples were collected in standard 3.8% sodium citrate and ethylenediaminetetraacetic acid (EDTA) tubes (BD, Madrid, Spain). Blood cell counts were performed with a Coulter AcT Diff cell counter (Beckman Coulter, Madrid, Spain).

Platelet-rich plasma (PRP) was obtained by whole citrated blood centrifugation (150 $\times$ *g* for 20 minutes at 23°C). To obtain washed platelets, the top two-thirds of the PRP volume were collected and centrifuged (650 $\times$ *g* for 10 minutes at 23°C) after adding acid-citrate-dextrose (1:10). The pellet was resuspended in an equal volume of HEPES buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl and 1 mM $MgSO_4$, pH 7.4).

Platelet-free plasma (PFP) was obtained by centrifuging at 1,500 $\times$ *g* for 15 minutes at 23°C, followed by 2 additional centrifugations (the first for 15 minutes at 1,500 $\times$ *g*; the second for 2 minutes at 13,000 $\times$ *g*). PFP aliquots were stored at –80°C until analysis.

All samples were analysed or stored properly within 2 hours of sampling.

Plasma Thrombopoietin

EDTA-anticoagulated whole blood was centrifuged at 1,000 $\times$ *g* for 15 minutes at 23°C to obtain platelet-poor plasma (PPP). PPP was stored at −80°C until the assay. Plasma TPO concentrations were determined by a commercially available enzyme-linked immunosorbent assay (ELISA; Quantikine; R&D Systems, Minneapolis, Minnesota, United States), according to the manufacturer's instructions.

Assay of Reticulated Platelets

Reticulated platelets were measured as previously described.[19] Briefly, after PRP preparation, washed platelets were fixed in 1% formaldehyde for 15 minutes at room temperature (RT) to minimize non-specific staining and resuspended in phosphate-buffered saline (PBS). Fifty microlitres of this suspension were mixed with 5 µL of phycoerythrin (PE)-αIIb monoclonal antibody (mAb) (BioCytex, Marseille, France) and incubated for 30 minutes at RT. Next, 600 µL of PBS and 100 µL of Retic-COUNT (thiazole orange; Becton Dickinson, San Jose, California, United States) were mixed and incubated for 2 hours at RT. Samples were analysed in a flow cytometer (FACScan flow cytometer, BD Biosciences, Madrid, Spain).

Determination of Platelet Activation

PRP was diluted 1:5 with HEPES buffer and incubated with or without 100 µmol/L thrombin receptor-activating peptide 6 (TRAP; Bachem, Switzerland) at RT. Following incubation, fluorescein isothiocyanate (FITC) anti-P-selectin mAb (BD Pharmingen, San Diego, California, United States), FITC anti-CD63 mAb (BD) FITC-PAC1 (BD), a mAb that recognizes activated conformation of fibrinogen receptor, was added for 15 minutes at RT. After incubation, platelets were diluted in HEPES buffer for flow cytometry analysis.

Rotational Thromboelastometry

Using a viscoelastometric clotting test (ROTEM, Pentapharm, Munich, Germany), we evaluated the kinetics of clot formation and fibrinolysis.

ROTEM was performed on fresh PRP adjusted to 25 $\times$ 10^9 platelets/L with PPP from the same participant. The samples were allowed to rest at RT for 30 minutes before testing. Recalcification (naTEM) was performed to assess the kinetics of clot formation. We recorded the clotting time (CT, time from the start of measurement to the start of clotting, in seconds); clot formation time (time from the start of clotting to 20 mm of amplitude, in seconds, which reflects the speed of the clotting process); α angle (tangent to the curve at 2 mm amplitude, in degrees, which reflects the rate of fibrin polymerization); amplitude at time 'x' (in mm); maximum clot firmness (MCF, in mm, which reflects the maximum tensile strength of the thrombus); and lysis at 60 minutes (LI60, in %; residual clot firmness 60 minutes after CT).

Measurement of Phosphatidylserine Exposure on Platelet Surface and Activity of Caspases

The surface exposure of phosphatidylserine (PS) in washed platelets was assessed by measuring the binding of FITC-labelled annexin V (BD Pharmingen, Madrid, Spain). Briefly, washed platelets were re-suspended in annexin V binding buffer (10 mM HEPES, 10 mM NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and labelled with FITC–annexin V. After incubation for 15 minutes at RT in the dark, samples were analysed by flow cytometry.

To analyse active caspase-3, -7, -8 or -9, PRP was diluted 10-fold with isotonic HEPES-buffered saline with Ca^{2+} (5 mM HEPES; 140 mM NaCl; 2.7 mM KCl; 0.42 mM NaH2PO4; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 12 mM $NaHCO_3$; 0.35% bovine serum albumin; 5 mM dextrose, pH 7.4) containing 2 mM Gly-Pro-Arg-Pro (Sigma-Aldrich, Madrid, Spain) to prevent fibrin formation. We then added PE-labelled mAb to αIIb and FAM-DEVD-FMK, FAM-LETD-FMK or FAM-LEHD-FMK (Millipore, Madrid, Spain) to the samples and analysed them by flow cytometry.

Determination of Microparticle Pro-Coagulant Activity

MP pro-coagulant activity was determined in PFP with the ZYMUPHEN MP-Activity kit (Hyphen BioMed, Neuville sur Oise, France) following the manufacturer's instructions. This kit is based on a pro-thrombinase assay after MP capture on a microtitration plate coated with streptavidin and biotinylated annexin V.

Pro-Thrombinase Complex Binding to Platelets

Quiescent washed platelets (1×10^8/mL) were incubated with activated factor X (FXa, 5 nmol/L) and FVa (5 nmol/L) obtained from Haematologic Technologies Inc. (CellSystems, Germany). Following fixation to cross-link platelet-bound FVa and FXa, specific or control monoclonal antibodies (0.1 µM each, Haematologic Technologies Inc., Germany) were added. After washing, FITC-conjugated polyclonal rabbit anti-mouse immunoglobulins (Igs; DAKO, Spain) were added. An additional aliquot was incubated with PE-αIIb mAb as control. After a 20-minute incubation period and washing, the samples were analysed by flow cytometry.

In a separate set of experiments and to evaluate whether the presence of antibodies may interfere with the binding of pro-thrombinase complex, washed platelets from healthy controls were incubated during 30 minutes with Abciximab (5 µg/mL, Janssen, Spain), an antibody that blocks fibrinogen receptor, before adding FVa and FXa.

ELISA Determinations

The human urokinase-plasminogen activator (uPA) kit used was from R&D Systems Europe Ltd. (Abingdon, United Kingdom); the tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) kits were obtained from eBioscience Ltd. (Hatfield, United Kingdom); and thrombin activatable fibrinolysis inhibitor (TAFI) activity was from American Diagnostica (Pfungstadt, Germany). All these substances were determined in PPP samples, following the manufacturer's instructions and measured in Multiskan FC with an incubator (Thermo Scientific, Madrid, Spain). In addition, PAI-1 was determined in washed platelets lysed by 5 freezing/thawing cycles.

Cell Culture and Modification of Protein Expression in MEG-01 Cells

MEG-01 cells (generously provided by Dr. Redondo, University of Extremadura, Cáceres, Spain) were maintained in Roswell Park Memorial Institute medium (Gibco, Madrid, Spain), supplemented with 10% foetal bovine serum (ICN, Madrid, Spain), 100 U/mL penicillin G (Gibco) and 100 µg/mL streptomycin (Gibco), at 37°C and 5% CO_2 (3×10^5 cells/mL). Three MEG-01 cell cultures were initiated simultaneously: control without drugs and stimulated by either TPO (100 ng/mL) or romiplostim (53 µg/mL). Samples were collected at the start and after 24, 48 and 72 hours to determine the PAI-1 content or 72 hours and 1 week to measure caspase-3, -7, -8 or -9 activity.

PAI-1 Content in MEG-01 Cells

The endogenous PAI-1 content in the MEG-01 cells was determined by Western blot. Washed cells were lysed with Triton buffer (1% Triton X-100, 0.05% Tween-20, 300 mM NaCl and 1 mM phenylmethylsulfonyl fluoride in PBS) containing a cocktail of protease inhibitors (Complete Mini, EDTA free; Roche, Barcelona, Spain). A total of 50 µg of MEG-01 protein lysates underwent sodium dodecyl sulphate-polyacrylamide gel electrophoresis in reduced conditions. Then, the proteins were transferred onto a nitrocellulose membrane with a Trans-blot semidry transfer cell (Bio-Rad, Madrid, Spain). Membranes were first blotted with mAb against PAI-1 and then against tubulin (both from Santa Cruz Biotech Inc, Santa Cruz, California, United States) as a sample charge control. The membranes were then incubated with an anti-mouse IgG antibody-conjugated with horseradish peroxidase. Membranes were revealed by enhanced chemiluminescence (Merck, Madrid, Spain), and densitometric analyses were performed with Alliance 2.7 imaging system (UVITEC Cambridge, United Kingdom).

Caspase Activity in MEG-01 Cells

Caspase activity was determined by flow cytometry using Millipore kits, as described above for platelets.

Statistics

To calculate minimum sample size, the G*Power software (Heinrich Heine, Dusseldorf University) was used. A minimum sample of 25 participants per group would provide 80% power at two tails to detect a difference between groups, assuming a 5% level of significance.

Experimental data were analysed using the SPSS 9.0 software (SPSS Inc., Chicago, Illinois, United States). The Shapiro–Wilk test was used to test the normal distribution. To compare multiple groups, one-way analysis of variance or non-parametric Kruskal–Wallis, using Dunn's multiple comparison post hoc test were performed. All tests were two-tailed, and the level of statistical significance was set at $p < 0.05$.

Results

Patient Population

Forty patients with ITP without treatment for at least 6 months (75% women, mean age of 51 ± 7 years) (►**Table 1**) and 42 patients with ITP responders to TPO-RA therapy (54.7% women, mean age of 64 ± 8 years; 64.3% with eltrombopag, 35.7% with romiplostim) (►**Table 2**) were included. Response to TPO-RA therapy was defined as reaching a platelet count of $> 30 \times 10^9$/L and at least twofold

Table 1 Features of ITP patients without treatment (UT-ITP)

No.	Age, years	Sex	Time since diagnosis, years	Previous treatments	Concomitant treatments	Platelet count ($\times 10^9$/L)
1	23	F	1.6	IVIgs	No	41
2	24	F	7.3	Corticosteroids, IVIgs	Ferrous sulphate	33
3	18	M	1.8	Corticosteroids, IVIgs	No	116
4	92	F	3.0	Corticosteroids, IVIgs	Carvedilol	45
5	49	F	3.2	No	No	54
6	55	F	17.6	Corticosteroids, IVIgs	No	17
7	80	F	2.2	Corticosteroids, IVIgs	Rabeprazole, Carvedilol, Tramadol hydrochloride/ Paracetamol, Simvastatin, Gliclazide, Candesartan, Tetrazepam, Ibuprofen, Calcium carbonate, Calcitriol, Bisphosphonates, Iron/sucrose injection	123
8	67	F	4.3	Corticosteroids, IVIgs	No	180
9	69	M	3.6	No	No	22
10	75	F	1.2	No	Hydrochlorothiazide, Irbesartan	38
11	45	F	3.1	No	No	158
12	61	F	17.3	Corticosteroids, IVIgs	Ranitidine	49
13	56	F	7.3	No	No	53
14	62	M	2.5	Corticosteroids, IVIgs	No	170
15	67	M	2.3	No	No	47
16	84	M	5.6	No	No	130
17	55	M	8.1	No	No	175
18	54	F	17.5	Corticosteroids, IVIgs	No	22
19	85	F	18	Corticosteroids, IVIgs	No	68
20	46	F	15.5	Corticosteroids, IVIgs	No	20
21	46	F	14.0	Corticosteroids, IVIgs	No	79
22	23	M	14.7	Corticosteroids, IVIgs	No	28
23	21	M	1.3	Corticosteroids, IVIgs	No	51
24	69	F	16.4	No	Rifaximin, Levothyroxine, Verapamil, Levocetirizine dihydrochloride, Bromazepam, Atorvastatin, Trimetazidine, Olmesartan medoxomil, Chlortalidone, Thiamine Hydrochloride+ Cyanocobalamin+ Pyridoxine Hydrochloride, Alendronic acid, Beclometasone dipropionate, Montelukast	32
25	76	F	21.0	No	No	37
26	75	F	1.0	No	Enalapril, Ezetimibe	53
27	26	F	4.1	No	No	53
28	40	F	15.9	Corticosteroids, IVIgs	No	243
29	40	F	1.5	No	No	139
30	44	F	2.1	No	No	89
31	46	F	1.8	Corticosteroids, IVIgs	No	33
32	42	F	1.2	No	No	33
33	35	F	5.3	Corticosteroids, IVIgs	No	66
34	36	F	1.7	Corticosteroids	No	107
35	25	F	4.6	Corticosteroids	Propranolol	50
36	79	M	24.8	Immunosuppressant drugs	No	109
37	49	M	1.6	No	No	95
38	54	F	1.0	No	No	68
39	40	F	1.3	Corticosteroids, Azathioprine	Diazepam, Clonazepam	146
40	35	F	8.7	No	No	149

Abbreviations: F, female; ITP, immune thrombocytopaenia; IVIgs, intravenous immunoglobulins; M, male; UT, untreated.

Table 2 Features of ITP patients on treatment with TPO-RAs

No.	Age, years	Sex	Time since diagnosis (years)	Current treatment (TPO-RA)	Time with current TPO-RA (mo)	Previous treatments	SPL (year)	Concomitant treatments	Platelet count ($\times 10^9$/L)
1	40	F	9	Eltrombopag 50 mg/d	20	IVIgs Corticosteroids	No	Lamivudine, Abacavir sulphate, Dolutegravir, Fluconazole, Spironolactone	32
2	30	M	20	Eltrombopag 25 mg/d	49	IVIgs Corticosteroids	No	No	55
3	77	M	25	Romiplostim 8 μg/kg/7 d	44	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to transaminase increase)	No	Metformin Ivabradine, Atorvastatin, Pantoprazole	45
4	47	F	6	Eltrombopag 50 mg/d	34	IVIgs Corticosteroids	No	Levothyroxine, Omeprazole	354
5	58	F	37	Romiplostim 10 μg/kg/7 d	55	IVIgs Eltrombopag (treatment was suspended due to lack of response)	Yes (1984)	Calcium Pidolate/ Cholecalciferol, Lorazepam	30
6	40	M	16	Romiplostim 4 μg/kg/7 d	28	IVIgs, Corticosteroids Eltrombopag (treatment was suspended due to lack of response)	No	Celecoxib, Tramadol hydrochloride/ Paracetamol, Omeprazole	133
7	45	M	9	Romiplostim 1 μg/kg/d	6	IVIgs Corticosteroids Rituximab Eltrombopag (treatment was suspended due to lack of response)	No	No	225
8	46	M	10	Romiplostim 1 μg/kg/d	19	IVIgs Corticosteroids Rituximab Eltrombopag (treatment was suspended due lack of response)	No	No	311
9	85	M	18	Romiplostim 10 μg/kg/2 d +IVIgs/2 d	30	IVIgs Corticosteroids	No	Enalapril	166
10	88	M	21	Eltrombopag 25 mg/d	19	IVIgs, Corticosteroids Romiplostim (treatment was suspended due to lack of response)	No	Enalapril, Darbepoetin alfa	226
11	43	M	41	Romiplostim 1 μg/kg/7 d	47	IVIgs Corticosteroids Azathioprine Rituximab, Eltrombopag (treatment was suspended due to loss of response)	Yes (1980)	Pegylated interferon, Ribavirin, Boceprevir	119
12	81	M	5	Prednisone 20 mg + Eltrombopag 25 mg/d	12	IVIgs Corticosteroids Rituximab	No	Perindopril, Paracetamol	281
13	71	M	14	Eltrombopag 25 mg/d	53	IVIgs Corticosteroids Azathioprine	No	No	109
14	72	M	15	Eltrombopag 75 mg/d	68	IVIgs Corticosteroids Dapsone Azathioprine	No	No	117
15	73	M	16	Romiplostim 10 μg/kg/7 d + Corticosteroids 10 mg/2 d	8	IVIgs Corticosteroids Dapsone Azathioprine Eltrombopag (treatment is suspended due to loss of response)	No	No	39
16	41	F	13	Eltrombopag 75 mg/d	23	IVIgs Corticosteroids	Yes (2013)	No	99

(*Continued*)

Table 2 (*Continued*)

No.	Age, years	Sex	Time since diagnosis (years)	Current treatment (TPO-RA)	Time with current TPO-RA (mo)	Previous treatments	SPL (year)	Concomitant treatments	Platelet count ($\times 10^9$/L)
17	75	F	12	Eltrombopag 75 mg/d	38	IVIgs Corticosteroids	No	No	57
18	76	F	13	Eltrombopag 75 mg/d	49	IVIgs Corticosteroids	No	No	40
19	78	M	6	Eltrombopag 25 mg/3 d	2	IVIgs Corticosteroids	No	Omeprazole, Metformin, Carvedilol, Paroxetine, Digoxin	351
20	75	M	16	Eltrombopag 25 mg/d	30	IVIgs Corticosteroids	No	Omeprazole, Celecoxib, Silodosin, Paracetamol	86
21	84	M	7	Eltrombopag 25 mg/3 d	15	IVIgs Corticosteroids	No	Atorvastatin, Pantoprazole, Ferrous sulphate	97
22	82	F	36	Romiplostim 8 μg/kg/7 d	39	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to hepatic toxicity)	No	Ferrous sulphate, Omeprazole, Candesartan, Atorvastatin, Timolol	60
23	18	F	6	Eltrombopag 25 mg/d	2	IVIgs Corticosteroids Romiplostim (treatment was suspended due to loss of response)	No	No	30
24	74	F	3	Romiplostim 2 μg/kg/2 d	2	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to hepatic toxicity)	No	Levothyroxine, Fluoxetine, Tramadol hydrochloride/Paracetamol, Lorazepam, Omeprazole, Enalapril, Calcifediol, Etoricoxib	116
25	60	F	23	Eltrombopag 25 mg/d	25	IVIgs, Corticosteroids	No	No	66
26	61	F	24	Eltrombopag 50 mg/d	40	IVIgs, Corticosteroids	No	No	172
27	75	F	3	Eltrombopag 50 mg/d	17	IVIgs Corticosteroids	No	Diltiazem, Paracetamol, Furosemide, Simvastatin, Allopurinol, Nitroglycerin, Cafinitrina, Lorazepam	260
28	85	F	29	Romiplostim 1 μg/kg/7 d	40	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to lack of response)	No	Calcifediol, Alendronate	93
29	66	F	14	Eltrombopag 50 mg/d	12	IVIgs Corticosteroids Rituximab	No	Diosmin, Bromazepam, Lorazepam	157
30	79	M	1	Eltrombopag 75 mg/d	12	IVIgs Corticosteroids	No	Magnesium	79
31	51	F	6	Eltrombopag 25 mg/d	28	IVIg, Corticosteroids	No	No	77
32	74	F	1	Romiplostim 6 μg/kg/7 d	7	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to lack of response)	No	No	146
33	62	M	13	Eltrombopag 75 mg/d	1	IVIgs Corticosteroids	Yes (2003)	Simvastatin, Allopurinol, Omeprazole, Metformin, Prednisone	110
34	64	M	3	Romiplostim 6 μg/kg/2 d	16	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to lack of response)	No	No	98
35	81	F	5	Eltrombopag 25 mg/3 d	11	IVIgs Corticosteroids	NO	Enalapril, Atorvastatin	73

Table 2 (*Continued*)

No.	Age, years	Sex	Time since diagnosis (years)	Current treatment (TPO-RA)	Time with current TPO-RA (mo)	Previous treatments	SPL (year)	Concomitant treatments	Platelet count ($\times 10^9$/L)
36	45	F	3	Eltrombopag 25 mg/ 3 d	17	IVIgs Corticosteroids	No	Atenolol, Ursodeoxycholic acid, Omeprazole	55
37	79	F	13	Eltrombopag 25 mg/d	54	IVIgs Corticosteroids Rituximab Romiplostim (treatment was suspended due to lack of response)	No	Omeprazole, Citalopram	260
38	81	M	8	Eltrombopag 50 mg/d	20	IVIgs Corticosteroids Rituximab	No	Perindopril, Paracetamol, Prednisona	281
39	79	F	4	Eltrombopag 25 mg/d	32	IVIgs Corticosteroids Rituximab	No	No	179
40	59	F	20	Romiplostim 8 µg/kg/7 d	24	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to lack of response)	No	Levothyroxine	57
41	47	F	6	Romiplostim 7 µg/kg/7 d	2	IVIgs Corticosteroids Eltrombopag (treatment was suspended due to lack of response)	No	No	359
42	77	F	14	Eltrombopag 25 mg/2 d	10	IVIgs Corticosteroids Dapsone	Yes (2003)	Olmesartan medoxomil/ Amlodipine, Captopril, Metformin	63

Abbreviations: F, female; ITP, immune thrombocytopaenia; IVIgs, intravenous immunoglobulins; M, male; SPL, splenectomy; TPO-RA, thrombopoietin receptor agonist.

increase in the baseline count and absence of bleeding.[1] One hundred and twelve healthy participants (55% women, mean age of 57 ± 25 years) were also recruited.

All patients treated with TPO-RA had previously been treated with corticosteroids and intravenous Ig, among other therapies, and five of them had undergone a splenectomy. None of them had history of thrombotic events.

Platelet Count, Immature Platelets and Thrombopoietin Plasma Levels

As shown in ►**Fig. 1A**, the platelet counts for the untreated (UT) and TPO-RA-treated groups were significantly lower than for the controls, whereas the proportion of immature platelets in all patients with ITP was higher (►**Fig. 1B**).

TPO plasma levels were higher in the patients with ITP than in the healthy controls, although statistical significance was only achieved in the TPO-RA-treated group (►**Fig. 1C**).

Global Haemostasis in Patients with Immune Thrombocytopaenia Responders to Thrombopoietin Receptor Agonists

The ROTEM studies showed significant differences in the dynamics of clot formation when comparing the control with ITP samples.

There was a delay in clot formation in the UT-ITP group, as observed by a prolonged CT (expressed as median [p25–p75]: control: 516 [490, 633] seconds; UT-ITP: 938 [914, 1348] seconds, $p < 0.001$), and a diminished α angle (control: 61.7 ± 5.6 degrees; UT-ITP: 49.2 ± 7.3 degrees, $p < 0.05$). Nevertheless, samples from patients with UT-ITP reached the same MCF as those from healthy controls (control: 45.3 ± 2.4 mm; UT-ITP: 46.9 ± 3.7 mm). On the other hand, patients with ITP undergoing TPO-RA therapy presented an initial clot formation similar to that of the control group (expressed as median [p25–p75]: CT, 672 (598, 928) seconds; α angle, 55.8 ± 5.8 degrees) but achieved a higher MCF (53.1 ± 4.5 mm, $p < 0.05$) and a reduced clot lysis after 60 minutes (control: 91.8 ± 4.0%; UT-ITP: 93.7 ± 4.0%, TPO-RA ITP: 97.6 ± 1.7, $p < 0.05$) (►**Fig. 2**).

Functional State of Platelets from Patients with Immune Thrombocytopaenia

Platelets from all patients with ITP had a defect in their ability to be activated, as shown by the lower PAC1 binding (►**Fig. 3A**) and surface exposure of P-selectin (►**Fig. 3B**) and CD63 (►**Fig. 3C**) after stimulation with TRAP. Nevertheless, patients with ITP undergoing therapy with TPO-RA showed an increased basal exposure of P-selectin (►**Fig. 3B**).

Microparticle-Associated Pro-Coagulant Activity in Patients with Immune Thrombocytopaenia Undergoing Therapy with Thrombopoietin Receptor Agonists

The patients with ITP on treatment with TPO-RA showed a significantly higher pro-coagulant capacity of MPs associated to PS than the control and UT-ITP groups (control, 5.5 ± 2.3 nmol/L; UT-ITP, 5.0 ± 2.8 nmol/L; TPO-RA, 11.9 ± 5.1 nmol/L, $p < 0.05$).

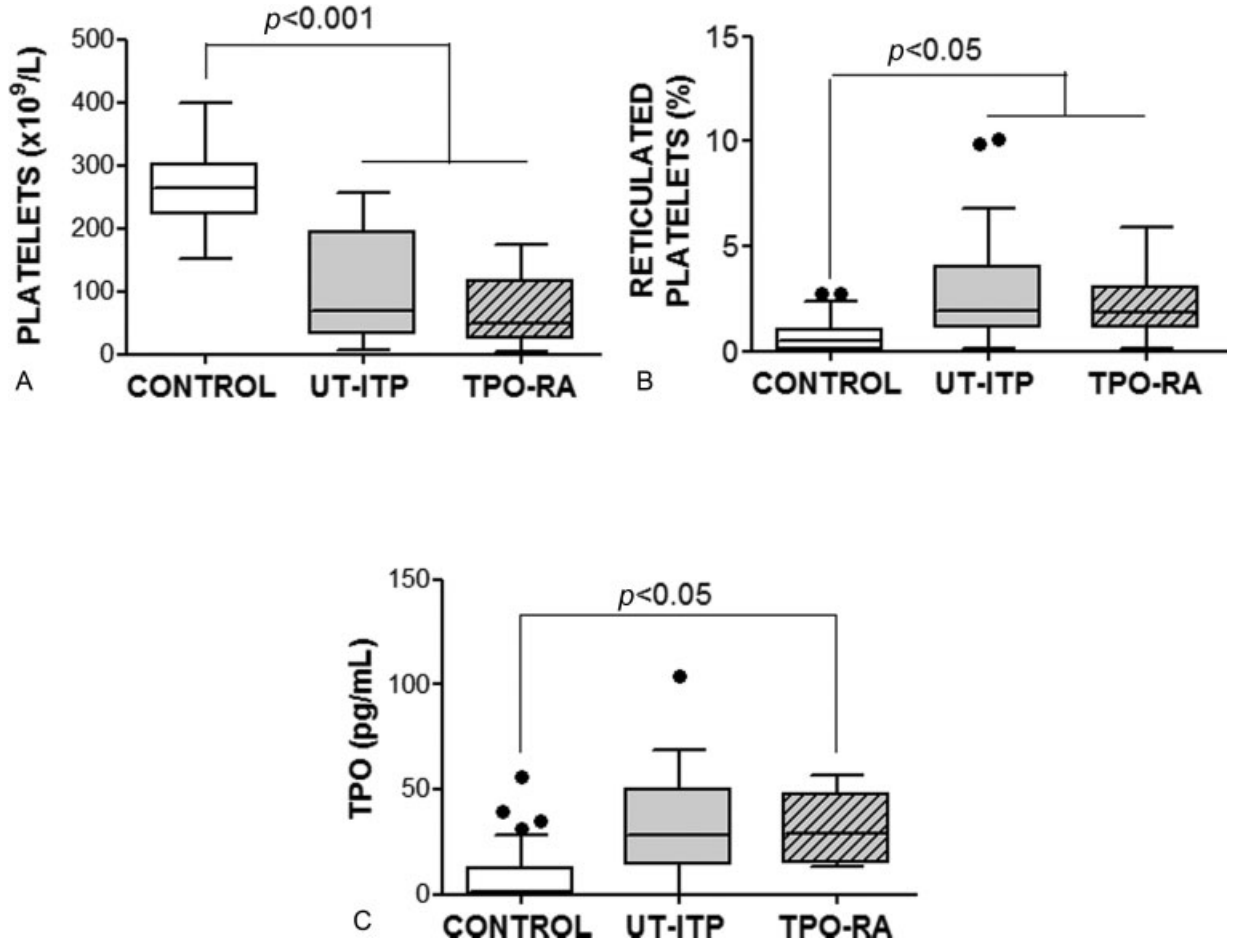

Fig. 1 Platelet count, immature platelets and thrombopoietin (TPO) plasma levels. (**A**) Platelet count in the three study groups: healthy participants (control), untreated patients with immune thrombocytopaenia (ITP) (UT-ITP) and those on treatment with TPO receptor agonists (TPO-RA). (**B**) Immature platelets determined by flow cytometry as referred in the 'Methods' section and shown as %. (**C**) Plasma TPO levels. Kruskal–Wallis and Dunn's multiple comparison tests were performed, and p-value of < 0.05 was considered significant.

Apoptosis in Platelets from Patients with Immune Thrombocytopaenia

Platelets from patients with ITP exposed more PS than controls, and this increase was higher in the group treated with TPO-RA (►**Fig. 4A**). This situation was accompanied by an increased activity of caspases-3, -7, -8 and -9 (►**Fig. 4B**).

Pro-Thrombinase Complex Binding to Quiescent Platelets

We evaluated whether PS was functional for anchoring the pro-thrombinase complex. Binding of FVa and FXa onto the platelet membrane surface was higher in quiescent platelets from patients with ITP undergoing therapy with TPO-RA (►**Fig. 4C**).

To evaluate whether the presence of antibodies may interfere with the binding of pro-thrombinase complex, the effect of pre-incubating platelets with Abciximab was tested. Presence of this antibody did not modify either binding of FVa (without Abciximab [%]: 29.1 ± 10.1, with Abciximab [%]: 33.1 ± 3.6) or binding of FXa (without Abciximab [%]: 36.9 ± 18.9, with Abciximab [%]: 37.3 ± 9.3).

Effect of Thrombopoietin Receptor Agonists on Caspases Activity in MEG-01 Cells

To elucidate whether increments in the platelet's caspase activity were due to TPO-RA therapy, the effect of TPO and romiplostim were tested on MEG-01 cells. These treatments significantly increased the activity of caspase-3, -7, -8 and -9 with a different temporal sequence (►**Fig. 5**).

Fibrinolysis in Patients with Immune Thrombocytopaenia Undergoing Therapy with Thrombopoietin Receptor Agonists

The increased LI60 values in patients with ITP undergoing therapy with TPO-RA might be due to an imbalance in fibrinolysis-related proteins. No intergroup differences were observed for tPA and uPA antigenic plasma levels and TAFI plasma activity (►**Table 4**). On the contrary, PAI-1 plasma levels were increased in patients with ITP treated with TPO-RA (►**Fig. 6A**). Since platelets are a source of PAI-1, we measured their endogenous content. As observed in ►**Fig. 6B**, PAI-1 was increased in the platelets from patients with ITP treated with TPO-RA.

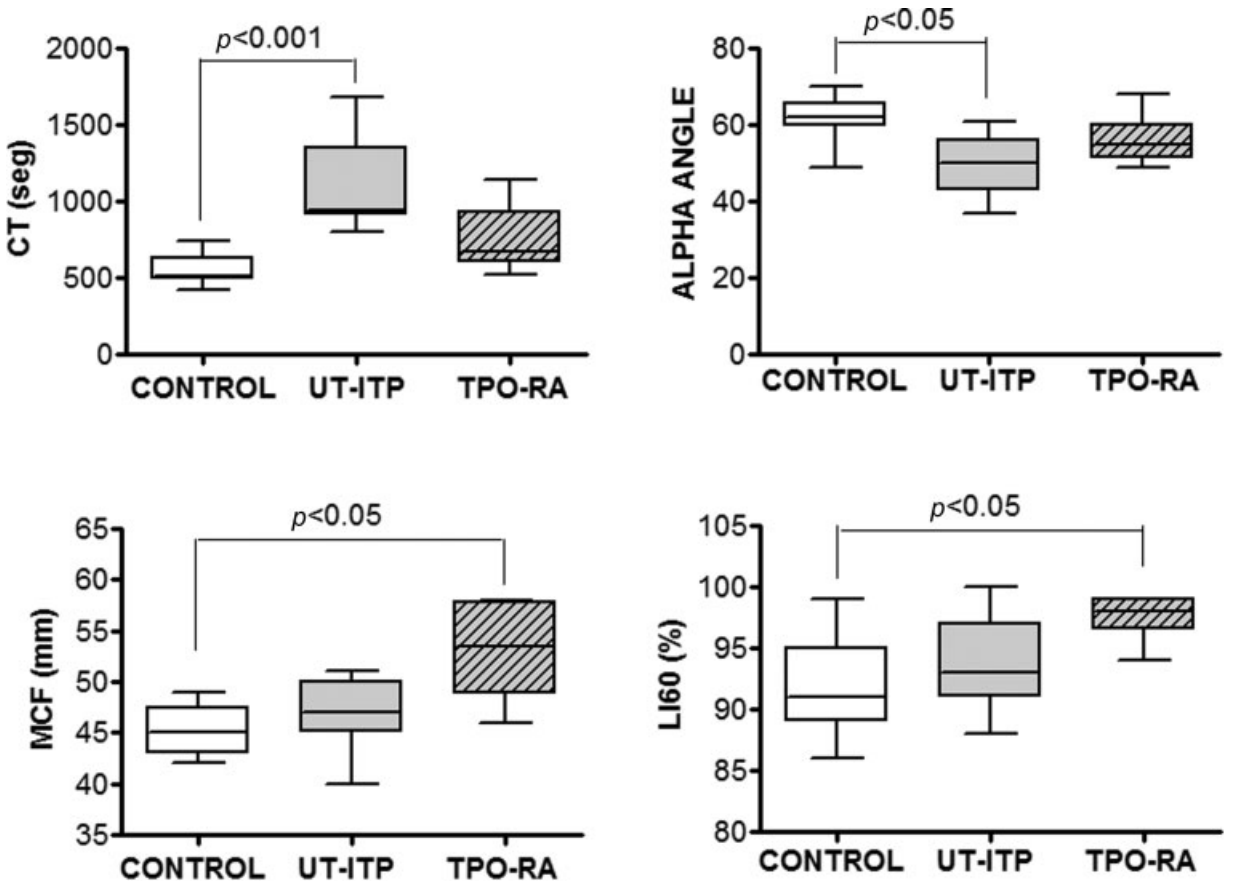

Fig. 2 Features of clot formation in patients with immune thrombocytopaenia (ITP). Thromboelastography was performed in platelet-rich plasma (PRP) adjusted to 25 $\times$ 10^9 platelets/L. Detailed procedures and measured parameters are explained in the 'Methods' section. Kruskal–Wallis and Dunn's multiple comparison tests were performed, and *p*-value of < 0.05 was considered significant.

To determine whether TPO-RA might be responsible for increased platelet PAI-1 content, experiments were performed in MEG-01 cells incubated with buffer, with TPO or with romiplostim. As shown in ►**Fig. 6C**, both drugs increased the endogenous content of PAI-1 but increment only reached significance after 48 hours of treatment.

Discussion

TPO-RA (romiplostim and eltrombopag) therapy is the most recent clinical development to change the landscape of second-line ITP therapy.[20] One of the concerns regarding TPO-RA is its small but significant increase in the risk of thrombosis.[14,15]

Our results show that the patients with ITP undergoing therapy with TPO-RA presented a pro-coagulant profile because of the formation of a more fibrinolysis-resistant clot due to increased PAI-1 levels, enhanced MP-associated pro-coagulant activity and increased platelet apoptosis, causing greater exposure of PS and, consequently, the availability of a larger surface for pro-thrombinase complex binding.

A recent meta-analysis of eltrombopag in ITP examined six randomized controlled trials demonstrating that eltrombopag significantly improved platelet counts and decreased the incidence of bleeding.[21] As reported in the literature, several patients who were either intolerant or refractory to one of the TPO-RA therapies successfully switched to the other.[22]

The results for a 3-year follow-up of patients in an open-label extension study for continued dosing of eltrombopag showed that the rate of thromboembolic events was 3.17 per 100 patient-years.[23] These data were recently updated at 5 years, with 6% of patients having thromboembolic events.[24]

Haselboeck et al[25] described the development of venous thromboses during eltrombopag therapy in 2 of 11 patients, with no association with any distinct single platelet function from the evaluated parameters. This might be because no measurements were performed for platelet PS exposure, the amount of MPs or the fibrinolytic system (present results).

For romiplostim, the rate of thromboembolic events was 5.5 per 100 patient-years for both romiplostim and placebo-treated patients.[26]

Large randomized clinical trials on ITP have shown that the occurrence of venous thrombosis was not related to platelet count or type or dose of TPO-RA.[27]

In any case, these thrombotic events reported for TPO-RA are lower than those reported for patients with ITP who underwent splenectomy.[28]

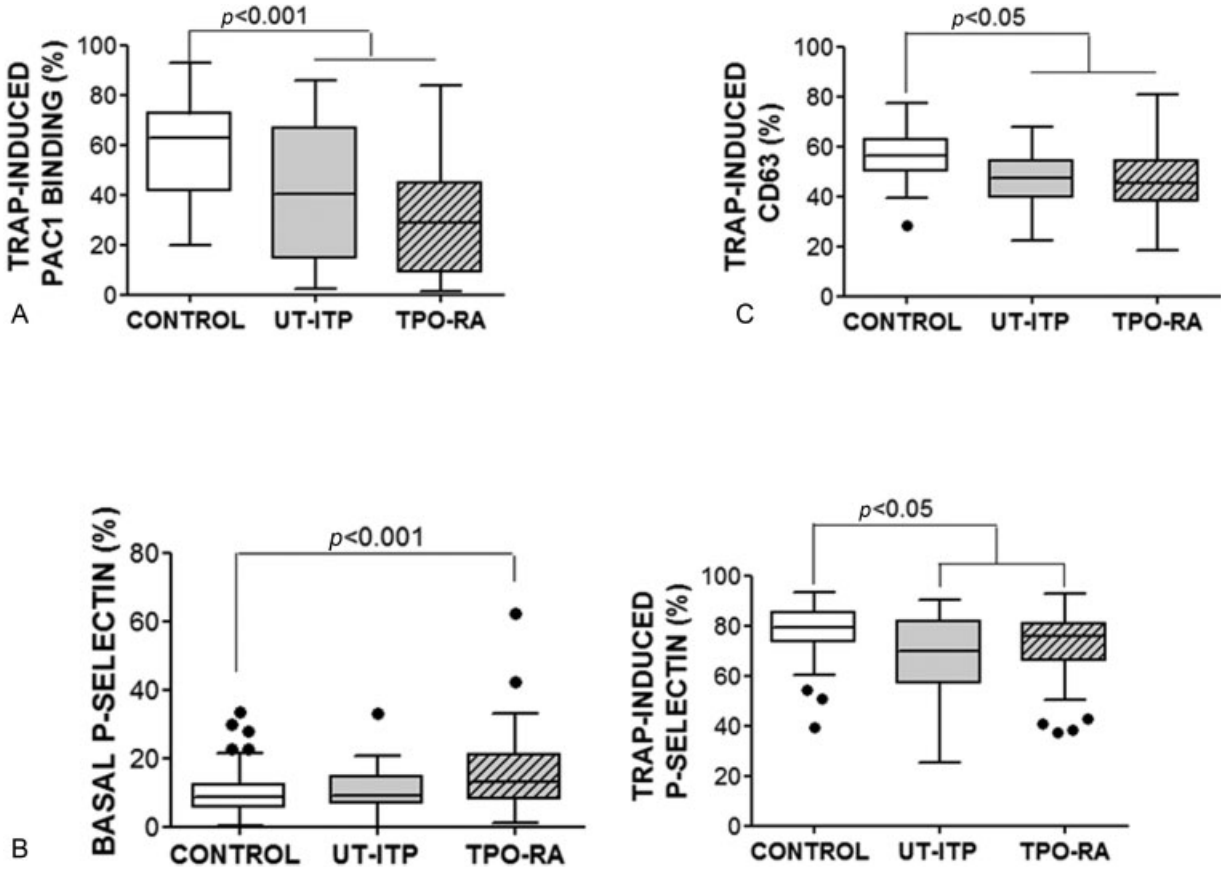

Fig. 3 Platelet activation markers. Platelets from healthy controls and from patients with immune thrombocytopaenia (ITP) (UT-ITP and thrombopoietin receptor agonist [TPO-RA]) in basal condition or stimulated with thrombin receptor-activating peptide (TRAP) were incubated with fluorescein isothiocyanate (FITC)-PAC1 (**A**), FITC-anti-P-selectin monoclonal antibody (mAb) (**B**) or FITC-anti-P-CD63 mAb (**C**) and analysed by flow cytometry. The data were expressed as % of positive cells. Kruskal–Wallis and Dunn's multiple comparison tests were performed, and *p*-value of < 0.05 was considered significant.

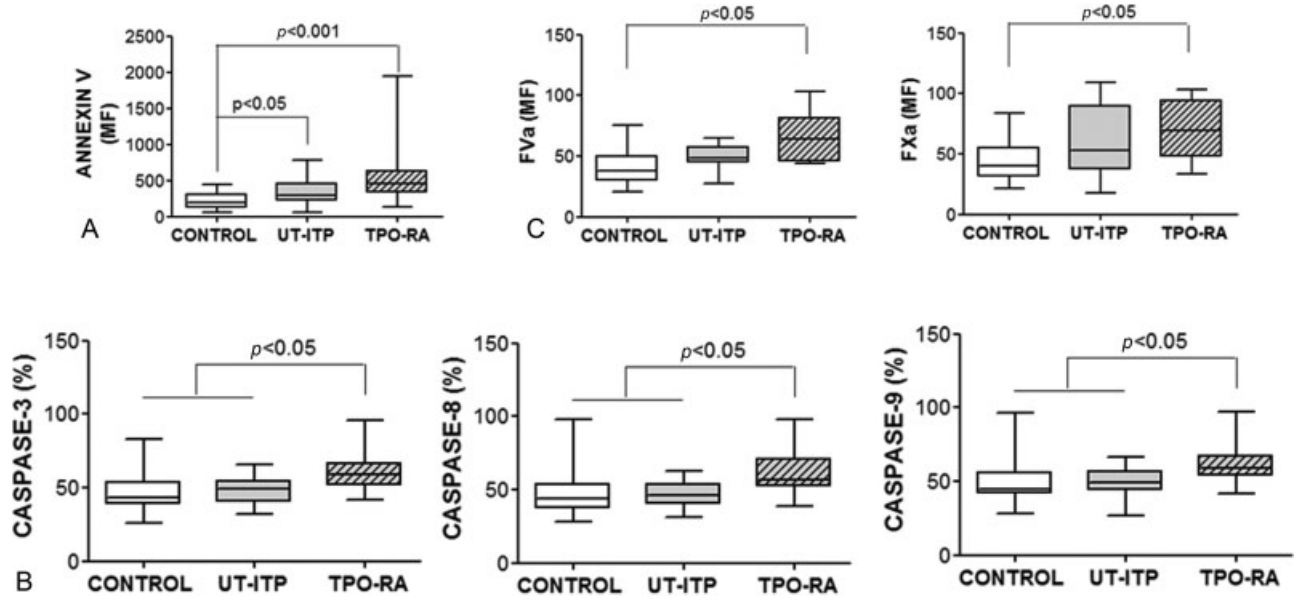

Fig. 4 Apoptosis signs and pro-thrombinase complex binding to platelets. Phosphatidylserine (PS) surface exposure determined by binding of fluorescein isothiocyanate (FITC)-annexin V (**A**), caspase activity (**B**) and factor Va (FVa) and FXa binding (**C**) in quiescent platelets from controls and patients with immune thrombocytopaenia (ITP) were determined by flow cytometry analysis. Data are expressed as mean fluorescence (MF, **A** and **C**) or % of positive cells (**B**). Kruskal–Wallis and Dunn's multiple comparison tests were performed, and *p*-value of < 0.05 was considered significant.

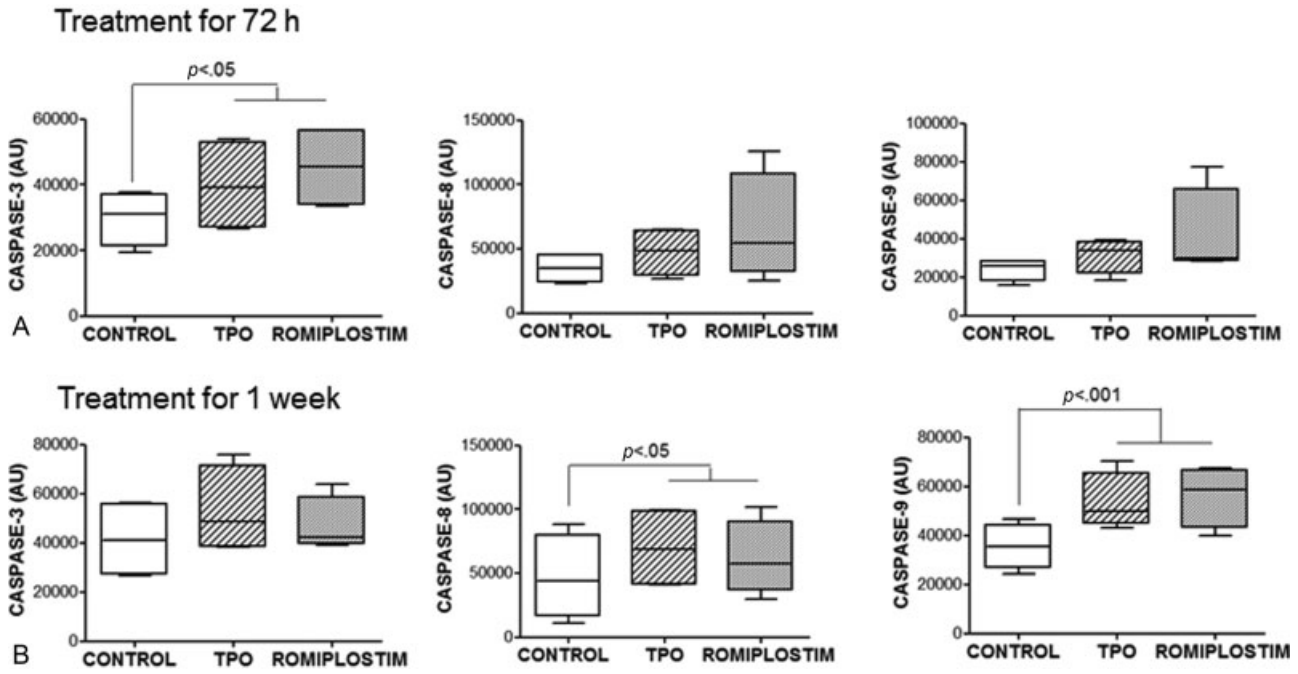

Fig. 5 Effect of recombinant thrombopoietin (TPO) and romiplostim on caspase activity in MEG-01 cells. MEG-01 cells were treated for 72 hours and 1 week without drugs (control) or with TPO (100 ng/mL) or with romiplostim (53 µg/mL). Activated caspases-3, -7, -8 and -9 were determined by flow cytometry as described in the 'Methods' section. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed, and *p*-value of < 0.05 was considered significant.

Table 3 Biochemical characteristics of patients with ITP

Biochemical parameters (units, range of normality)	Control % anomalous results		UT-ITP % anomalous results		TPO-RA % anomalous results	
	% lower	% higher	% lower	% higher	% lower	% higher
C3 (mg/dL, 75–135)	0	10	0	10	1	13
C4 (mg/dL, 14–60)	0	0	10	0	12	0
IgG (mg/dL, 725–1900)	0	0	0	13	2	11
IgM (mg/dL, 45–280)	0	0	0	0	0	7
IgA (mg/dL, 50–340)	0	10	0	8	0	26
LDH (UI/L, 100–190)	0	40	0	33	0	67
CRP (mg/L, 0.00–5.00)	0	10	0	14	0	20

Abbreviations: CRP, C-reactive protein; Ig, immunoglobulin; ITP, immune thrombocytopaenia; LDH, lactate dehydrogenase; TPO-RA, thrombopoietin receptor agonist; UT, untreated.
Note: Data are expressed as percentage of patients with anomalous results in each group (percentage below lower limit/percentage above the upper limit).

Table 4 Fibrinolysis-related proteins

	uPA, pg/mL	TAFI, % of activity	tPA, pg/mL
Control	658.2 ± 181.2	32.9 ± 4.9	641.1 ± 425.8
UT-ITP	911.7 ± 309.5	29.4 ± 6.2	665.4 ± 184.6
TPO-RA	620.6 ± 11.03	34.3 ± 7.2	708.7 ± 130.5

Abbreviations: ANOVA, analysis of variance; TAFI, thrombin activatable fibrinolysis inhibitor; tPA, tissue plasminogen activator; TPO-RA, thrombopoietin receptor agonists; uPA, urokinase-plasminogen activator; UT-ITP, untreated patients with immune thrombocytopaenia.
Note: Data are expressed as mean values ± standard deviation. The ANOVA test was performed and none were significant.

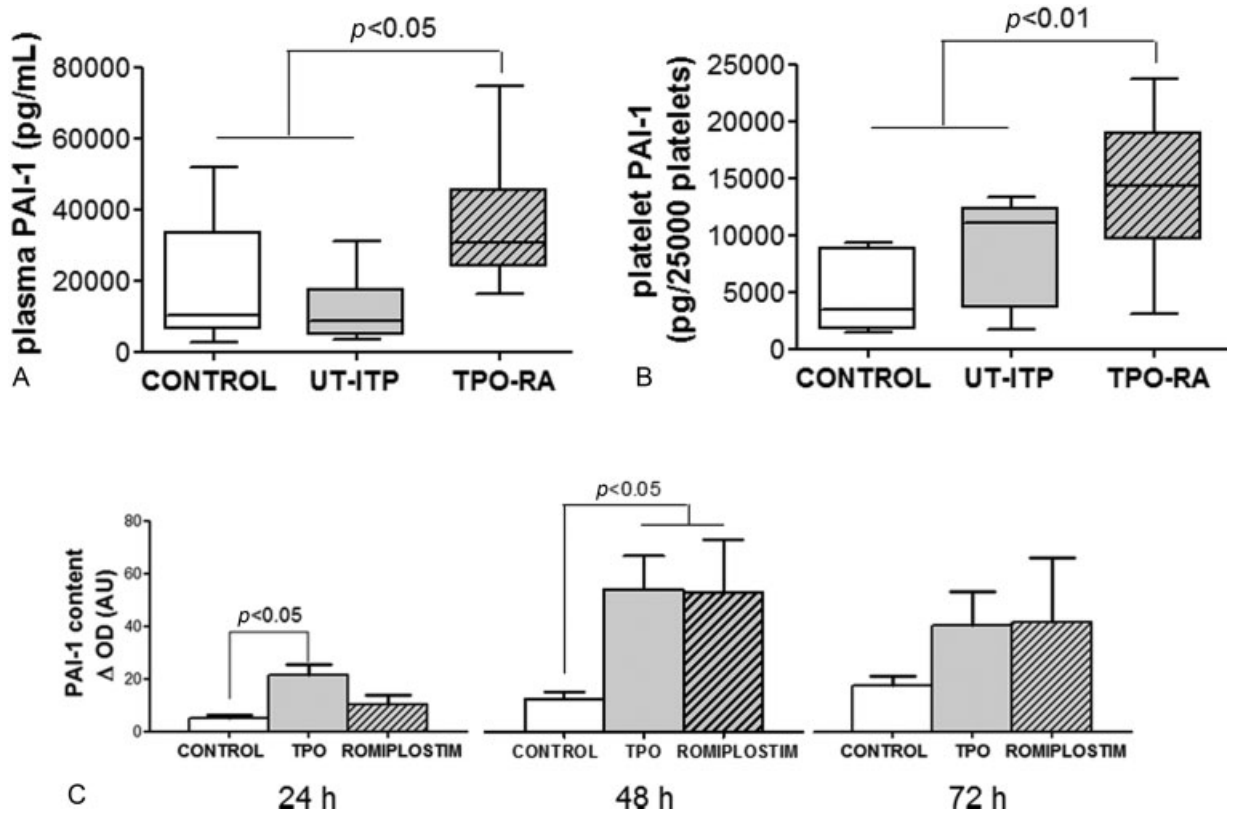

Fig. 6 Plasminogen activator inhibitor-1 (PAI-1) levels in plasma, platelets and MEG-01 cells. Plasma levels (**A**) and platelet content (**B**) of PAI-1 were measured by enzyme-linked immunosorbent assay (ELISA). (**C**) The effect of incubating MEG-01 cells without drugs (control) or with thrombopoietin (TPO) (100 ng/mL) or with romiplostim (53 µg/mL) for 72 hours and 1 week on PAI-1 expression was tested by Western blot as described in the 'Methods' section. Kruskal–Wallis and Dunn's multiple comparison tests were performed, and *p*-value of < 0.05 was considered significant.

ROTEM analyses of haemostasis showed that patients with UT-ITP had a delay in clot formation that was overcome in TPO-RA-treated patients. Moreover, this group showed increased MCF and hypo-fibrinolysis. The effect of TPO-RA on MCF might be related to platelet reactivity. A study reported that immature platelets had increased in vitro thrombogenicity,[29] but this finding does not seem to be related to the prothrombotic profile observed in TPO-RA-treated patients because both the ITP groups had similar immature platelet counts. Neither group seems to be related to the fibrinogen receptor's ability to be stimulated because it is impaired to the same extent in platelets from both groups. Since platelets from TPO-RA-treated patients in quiescent conditions exposed more P-selectin on their surface than untreated patients with ITP and healthy controls, there is the possibility of an enhanced interaction with another blood cell type.[3,30] Frelinger et al also reported that platelets from children with ITP undergoing a variety of therapies had increased P-selectin exposure in quiescent conditions but a reduced exposure after adenosine diphosphate (ADP) stimulation. These authors hypothesized that the fibrinogen receptor's reduced activation ability and capability to release P-selectin from granules might be a consequence of either the presence of platelet autoantibodies that cause platelet activation and release of platelet granule contents[31] or a desensitization of the platelet response to ADP. This latter explanation seems to be the most plausible because we did not find activated platelets in basal conditions, and approximately 30 to 40% of the cases had no detectable anti-platelet antibody levels. Garabet et al[32] also observed higher plasma soluble P-selectin levels and proposed its contribution to the increased risk of thrombotic events.

Platelets from patients with ITP had more apoptosis signals than those from healthy controls.[33] Externalization of PS onto the surface of cells undergoing apoptosis represents an 'eat me' signal that leads to the clearance of apoptotic platelets and worsens thrombocytopaenia.[34,35] Our results agree with Deng et al's observation and showed that platelets from patients with ITP on treatment with TPO-RA had enhanced apoptosis compared with those from UT-ITP patients. In contrast to our results, Mitchell et al[36] reported that TPO-RA therapy of patients with ITP induced platelet resistance to apoptosis and, consequently, to the extension of their circulating life span. This difference might be explained by the fact that this effect lasts for only 14 days and patients included in our cohort were undergoing TPO-RA therapy for longer than 2 weeks. This observation suggests that the effect of TPO-RA is dependent on the extent of the treatment. If this were the case, one of the limitations of our study could be different time our cohort of ITP patients have been receiving TPO-RA treatment. It would be interesting to perform a longitudinal study in a cohort of ITP patients taking samples before starting TPO-RA treatment until at least 3 to 6 months after to test time-dependence of TPO-RA

effect on platelet apoptosis. Goette et al[37] reported no differences between ITP and control platelets either in basal PS exposure or in caspase-3 activity, but an enhanced response to an apoptotic stimulus in platelets from patients with ITP. Moreover, these authors observed a decline in platelet apoptosis in 3 patients undergoing therapy with eltrombopag. As the authors discussed, this effect might be due to less sensitivity to platelet apoptosis and activation of the pro-survival Akt signalling pathway observed during the first week of TPO-RA therapy.[36]

PS exposed at the surface of platelets provides a negatively charged scaffold for the binding of calcium-dependent coagulation enzymatic complexes and provides a catalytic surface for the tenase and/or prothrombinase complexes that promote thrombin generation.[38] In support of a functional role for exposed PS, we observed increased binding of FVa and FXa in platelets from patients undergoing TPO-RA therapy. Moreover, patients with ITP undergoing therapy with TPO-RA had higher pro-coagulant capacity associated with PS from released MPs.

To evaluate whether the TPO-RA effect on platelets might be exerted at the megakaryocyte level, we tested the effects of romiplostim on MEG-01 cells. MEG-01 is a highly undifferentiated human megakaryoblastic cell line that can be easily matured to platelet-like particles.[39] We observed that TPO and romiplostim induced MEG-01 cell maturation due to a time-dependent increase in caspase activity. Similar results were reported by López et al,[40] with increased caspase-9 activity in MEG-01 that reached its peak after 6 days of TPO therapy.

The hypo-fibrinolysis observed in our study could be due, at least in part, to the increased PAI-1 levels. The patients with ITP treated with TPO-RA had higher plasma PAI-1 levels than the healthy controls. Similar results were reported by Garabet et al;[32] however, these authors failed to detect the plasma levels in the healthy controls, making it more difficult to interpret their results despite having performed them with the best analyses possible under their circumstances. Platelets have been identified as a major storage area for plasma PAI-1, accounting for more 90% of circulating PAI-1.[41] Platelets contain considerable amounts of translationally active messenger ribonucleic acid (mRNA) for PAI-1, and there is an on-going de novo PAI-1 synthesis in an active configuration.[42]

Our results showed a higher content of PAI-1 in platelets from TPO-RA-treated patients. The possibility that TPO and their RAs might increase PAI-1 mRNA levels in megakaryocytes is supported by the present results (the effect of TPO and romiplostim on MEG-01 cells) and by a previous study that showed that TPO increased PAI-1 levels in a megakaryocytic leukaemia cell line (UT-7) and in TPO-differentiated human cord blood $CD34^+/CD41^-$ cells to $CD34^+/CD41^+$ cells.[43]

Considering that the goal of ITP therapy is to prevent episodes of bleeding, additional pro-coagulant mechanisms involved in maintaining haemostasis in patients treated with TPO-RA might be considered a beneficial factor rather than a detrimental one that does not require the recovery of a normal platelet count. Nevertheless, in the presence of co-morbidities (e.g. advanced age, smoking, oral contraception and lifestyle considerations), these pro-thrombotic events might be taken under consideration for the choice of the therapeutic agent and its dose. Our results suggest that measurement of global haemostasis, thrombogenic capacity of MPs and fibrinolysis should be incorporated in clinical practice as a step towards a patient-oriented, precision medicine approach.

What is known about this topic?

- TPO-RAs have been successfully employed as second-line therapy for ITP.
- TPO-RAs have a small but significant increase in the risk of thrombosis.

What does this paper add?

- The patients with ITP undergoing TPO-RAs treatment presented a pro-coagulant profile due to the formation of a more fibrinolysis-resistant clot because of increased plasma and platelet PAI-1 levels.
- Moreover, platelets from TPO-RA-treated ITP patients presented an increased apoptosis that causes a higher exposure of phosphatidylserine and, consequently, a larger surface for the binding of the pro-thrombinase complex.
- Hypercoagulability caused by TPO-RAs should be taken into account when deciding which platelet count should be reached to avoid either haemorrhagic or thrombotic complications in ITP patients.

Authors' Contributions

R.J.S., E.M.M. and I.F.B. performed the experiments. M.T.A.R., M.M.S., M.I.R.P. and V.J.-Y. diagnosed, provided clinical care and verified patient information of immune thrombocytopaenia patients. All authors analysed the results. N.V.B. was the principal investigator, designed the experiments and wrote the manuscript. All authors approved the manuscript.

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Conflict of Interest

N.V.B. holds a Miguel Servet II tenure track grant from FIS-FONDOS FEDER (CP14/00024). N.V.B., M.T.A.R., I.F.B., M.M.S. and M.I.R.P. are paid instructors at Novartis. N.V.B. and M.T.A.R. are consultants for Novartis.

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Factors Involved in Maintaining Haemostasis in Patients with Myelodysplastic Syndrome

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Thromb Haemost

Abstract

Etiopathogenesis of myelodysplastic syndrome (MDS) might cause per se an anomalous haemostasis that can be even more deteriorated by thrombocytopaenia. So, evaluation of haemostasis in patients with MDS rises as a necessity.

This work aimed to characterize haemostasis in non-bleeder MDS patients with a platelet count similar to healthy controls to establish differences between the two groups not related to thrombocytopaenia.

Thromboelastometry in samples from MDS patients suggested the existence of at least two antagonistic processes: one of them giving a hypocoagulable pattern (prolonged clotting time and lower α angle) and another conferring a procoagulant profile (decreased fibrinolysis). Hypocoagulable state might be due to a decreased ability of platelets to be stimulated and to the presence in plasma of a factor/s that prolonged the time to initiate thrombin generation. This factor/s might be antibodies as this effect was observed in samples from MDS patients with an associated autoimmune-inflammatory condition.

Otherwise, hypercoagulable state seemed to rely on an increased presence of red cell- and monocyte-derived microparticles and to the increased exposure of phosphatidylserine that served as scaffold for binding of coagulation factors.

We concluded that haemostasis in MDS patients is a complex process influenced by more factors than platelet count.

Keywords
- platelets
- microparticles
- thromboelastometry
- MDS
- prothrombinase complex

Introduction

Myelodysplastic syndrome (MDS) comprises a heterogeneous sort of disorders caused by cytogenetic changes, gene mutations or both[1] responsible for altered multilineage morphological patterns that lead to a varying grade of peripheral blood cytopaenias and an increased risk of transformation to acute myeloid leukaemia. This ineffective haematopoiesis is caused by an anomalous relationship between haematopoietic progenitors and bone marrow microenvironment that promotes the formation and penetrance of a dysplastic clone. It has been recently reported that a great number of immunophenotypic alterations in bone marrow and in peripheral blood cells involve myeloblasts, $CD34^+$ B cell and $CD34^-$ precursors[2] and neutrophil, monocytic and erythroid cells.[3] Moreover, an increased apoptosis of haematopoietic progenitors that might be due to an immune response against the MDS clone mediated by cytotoxic T cells (CTC) and amplified by $CD4^+$ $CD25^+$ regulatory (Treg)–Th17 imbalances has been described.[4]

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It is widely accepted that subjects with platelet counts higher than 50,000/μL hardly bleed and that major haemorrhage is more likely at platelet counts below 10,000/μL. Nevertheless, some patients with severe thrombocytopaenia do not suffer from spontaneous bleeding. Thrombocytopaenia is observed in approximately 35% of MDS patients. We have recently reported that platelets from MDS patients had a diminished ability to be activated even in patients with normal or elevated platelet counts possibly due to the exacerbated platelet apoptosis observed.[5] In spite of this, it is estimated that less than 10% of MDS patients initially present serious haemorrhagic complications[6] and that only 9.8% of deaths in these patients were related to bleeding.[7] The precise platelet levels at which MDS patients become at risk of bleeding have not been well defined.[8] So, haemostasis in MDS patients is a complex issue where mechanisms additional to platelet count/function seem to be involved. This scenario turns even more complicated by comorbidities suffered by this mostly elder population and by therapeutic approaches used for MDS management. So, evaluation of haemostasis in MDS patients rises as a necessity.

This work aimed to characterize haemostasis in non-bleeder MDS patients with a platelet count similar to healthy controls to establish differences between the two groups not related to thrombocytopaenia. Recently, rotational thromboelastometry (ROTEM) emerged as a valuable tool to evaluate haemostasis in many pathological situations such as liver disease[9] and immune thrombocytopaenia (ITP).[10] So, ROTEM was employed to test haemostasis in our cohort of MDS patients.

Methods

Patients and Study Design

This is a prospective, observational and transversal study. Eighty-four non-bleeder MDS patients (44% female, age [mean ± SD] of 77 ± 25 years), whose features are described in ►**Table 1**, were included. Sixty healthy individuals (45% female, age [mean ± SD] of 57 ± 22 years) were recruited in the Blood Donor Section of the Hematology Unit of La Paz University Hospital.

Patients with uncontrolled hypertension, hyperlipidaemia, peripheral or coronary artery disease, abnormal hepatic or renal function tests or on treatment with platelet active drugs, lenalidomide, hydroxycarbamide, azacitidine or those who had received a transfusion within 15 days of the study were excluded.

The La Paz University Hospital Ethics Committee approved the experimental protocol. The research was performed in compliance with the Helsinki Declaration and after receiving signed patient-informed consent.

Preparation of Platelet-Rich Plasma and Washed Platelets

Human peripheral blood samples were collected in 3.8% sodium citrate. Blood cell counts were performed with a Coulter Ac.T Diff cell counter (Beckman Coulter, Madrid, Spain).

Table 1 Clinical features and therapies of MDS patients

WHO Classification	RARS	19 (22.5%)
	RAEB-1	5 (6.0%)
	RAEB-2	5 (6.0%)
	RCMD	36 (42.9%)
	RCUD	11 (13.1%)
	CMML	8 (9.5%)
IPSS	Low	56 (66.7%)
	Intermediate-1	26 (31%)
	Intermediate-2	1 (1.1%)
	High	1 (1.1%)
Cytopaenias	None	4 (4.8%)
	One	53 (63.1%)
	Two	26 (31.0%)
	Three	1 (1.1%)
Cytogenetic abnormalities	46,XY[20]	30 (35.7%)
	46,XX[20]	27 (32.1%)
	del(5q)	6 (7.1%)
	del(20q)	2 (2.4%)
	46,XY del(5)(q21q33)[17]	2 (2.4%)
	46,XX,del(20)(q12)	2 (2.4%)
	46,XX,i(14)(q10) [9]	1 (1.2%)
	46,XX (11q23)	1 (1.2%)
	47,XX, + 21	1 (1.2%)
	47,XY, + 8	1 (1.2%)
	47,XX, + 8 [9] 47, XX, + 8,i(17)(q10) [9]	1 (1.2%)
	46,XY,-18, + r(?) [21]	9 (10.7%)
	46,XX,add(3)(q29)	1 (1.2%)
AIC	Rheumatic polymyalgia	1 (1.2%)
	Psoriasis	2 (2.4%)
	Rheumatoid arthritis	6 (7.1%)
	Vasculitis	1 (1.2%)
	IgA-nephropathy	1 (1.2%)
	Pigmented purpuric dermatoses	1 (1.2%)
	Hypothyroidism	3 (3.6%)
	ASCA	1 (1.2%)
	ANA antibodies	2 (2.4%)
	ANCA	1 (1.2%)
	CRP	18 (21.4%)
	Rheumatoid factor	8 (9.5%)

(*Continued*)

Table 1 (*Continued*)

Treatment	Dried ferrous sulfate	1 (1.2%)
	Folic acid	6 (7.1%)
	None	45 (53.6%)
	Deferasirox	7 (8.3%)
	Erythropoietin	25 (29.8%)
	G-CSF	1 (1.2%)

Abbreviations: 5q SD, 5q-syndrome; AIC, autoimmune-inflammatory accompanying condition; ANA, antinuclear antibodies; ANCA, anti-neutrophil cytoplasmic antibodies; ASCA, anti-*Saccharomyces cerevisiae* antibodies; CMML, chronic myelomonocytic leukemia; CRP, C-reactive protein; IPSS, International Prognostic Scoring System; RA, refractory anaemia; RAEB-1, refractory anaemia with excess blast type 1; RAEB-2, refractory anaemia with excess blast type 2; RARS, refractory anaemia with ringed sideroblasts; RCMD, refractory cytopaenia with multilineage dysplasia; RCUD, refractory cytopaenia with uni-lineage dysplasia; WHO, World Health Organization.

Platelet-rich plasma (PRP) was obtained by centrifugation of whole blood (150 *g* for 20 minutes at 23°C). To obtain washed platelets, the top two-thirds volumes of PRP were collected and centrifuged (650 *g* for 10 minutes at 23°C) after the addition of acid–citrate–dextrose (ACD, 1:10). The pellet was resuspended in an equal volume of HEPES buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl and 1 mM $MgSO_4$, pH 7.4).

Platelet-poor plasma (PPP) was obtained by centrifugation at 1,500 *g* for 15 minutes at 23°C. Platelet-free plasma (PFP) was obtained by two additional centrifugations (first: 15 minutes at 1,500 *g*; second: 2 minutes at 13,000 *g*). PPP and PFP aliquots were stored at −80°C until analysis.

All the samples were analysed or stored properly within 2 hours of sampling as recommended to avoid significant artefacts in platelet analysis[11] and the release of cell microparticles (MPs) due to storage.[12]

Determination of Platelet Activation

Platelet-rich plasma was diluted 1:5 with HEPES buffer and incubated with 100 µmol/L thrombin receptor-activating peptide 6 (TRAP, Bachem, Switzerland) at room temperature (RT). Following incubation, fluorescein-isothiocyanate (FITC)–PAC1 (BD, Madrid, Spain), a monoclonal antibody (mAb) that recognizes activated conformation of fibrinogen receptor or FITC-labelled antihuman P-selectin mAb (BD Pharmingen, San Diego, California, United States) was added for 15 minutes at RT. After incubation, platelets were diluted in HEPES buffer for flow cytometry analysis with a FACScan flow cytometer (BD Biosciences, Madrid, Spain).

Calibrated Automated Thrombogram

Thrombin generation was measured in PPP by calibrated automated thrombogram (CAT) as described previously.[13] Coagulation was triggered by proper recalcification and the addition (final concentrations) of 1 pmol/L of recombinant human tissue factor and 4 µmol/L of phospholipid mixture (PPP-Reagent LOW, Thrombinoscope BV, Maastricht, the Netherlands). Lag time (LT = time when 10 nmol/L thrombin is formed); time-to-peak (TTP = time to reach the maximum thrombin concentration); peak height (PH = maximum thrombin concentration reached) and endogenous thrombin potential (ETP = area under the thrombin-concentration vs. time curve) were calculated with the Thrombinoscope software package (Thrombinoscope BV).

The effect of plasma from patients with MDS on thrombin generation of healthy control platelets was tested. To do so, washed platelets were resuspended in HEPES buffer, pooled and divided in aliquots containing 25×10^6 platelets which were additionally centrifuged (650 *g* for 3 minutes) after adding ACD 1:10. Platelet pellet was resuspended in 1 mL of PPP from the controls or the patients with MDS. Coagulation was triggered by platelet stimulation with 100 µmol/L TRAP.

Rotational Thromboelastometry

Kinetics of clot formation and fibrinolysis was evaluated with a viscoelastometric clotting test (ROTEM; Pentapharm, Munich, Germany).

ROTEM was performed on fresh PRP adjusted to 25×10^9 platelets/L with PFP from own subject. The samples were allowed to rest at RT for 30 minutes before testing. Only recalcification (NATEM test) was performed to assess the kinetics of clot formation. Clotting time (CT= time from start of measurement until initiation of clotting, in seconds); clot formation time (CFT = time from initiation of clotting to 20 mm amplitude, in seconds, which reflects the speed of the clotting process); α angle (tangent to the curve at 20 mm amplitude, in degrees, which reflects the rate of fibrin polymerization); amplitude at 'x' time (Ax, in mm); maximum clot firmness (MCF = maximum clot firmness, in mm, which reflects the maximum tensile strength of the thrombus) and lysis at 60 minutes (LI60) were recorded.

Determination of Cellular Origin and Procoagulant Activity of Microparticles

Phosphatidylserine (PS)-associated and tissue factor (TF)–associated procoagulant activity of MPs was determined with the ZYMUPHEN MP-Activity kits (HYPHEN BioMed, Neuville-sur-Oise, France).

The identification of the MP's cellular origin was determined by flow cytometry, labelling MPs with FITC-Annexin-V and the following specific mAb conjugated with phycoerythrin: anti-CD41 mAb for platelets (Biocytex; Marseille, France); and anti-CD14 mAb for monocytes, anti-CD144 mAb for endothelial cells, anti-CD235 mAb for red cells and anti-CD45 mAb for leukocytes, all from BD Biosciences.

Measurement of Phosphatidylserine Exposure on Platelet Surfaces and Caspases Activities

Surface exposure of PS in washed platelets was assessed by measuring the binding of FITC-labelled Annexin V (BD Pharmingen). Briefly, washed platelets were resuspended in Annexin V binding buffer (10 mM Hepes, 10 mM NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and labelled with FITC-Annexin V. After incubation for 15 minutes at RT in the dark, samples were analysed by flow cytometry.

To analyse active caspase-3, -8 or -9, PRP was diluted 10-fold with isotonic HEPES-buffered saline with Ca^{2+} (150 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM Gly-Pro-Arg-Pro [SIGMA; Madrid, Spain], 2 mM HEPES, pH 7. 4), containing the PE-labelled mAb to α2b, and either FAM-DEVD-FMK, FAM-LETD-FMK or FAM-LEHD-FMK (Millipore; Madrid, Spain).

Prothrombinase Complex Binding to Platelets

Either quiescent or 100 µM TRAP stimulated washed platelets (1×10^8/mL) were incubated with factor (F) Xa (5 nmol/L) and FVa (5 nmol/L) obtained from Haemtech Inc (Cellsystems, Germany). Following fixation to cross-link platelet-bound FVa and FXa, specific or control monoclonal antibodies (0.1 µmol/L each; Haemtech Inc, Germany) were added. After washing, FITC-conjugated polyclonal rabbit anti-mouse immunoglobulin (DAKO, Spain) was added. An aliquot was incubated with phycoerythrin (PE)-mAb against the αIIb subunit (Biocytex; Marseille, France). All samples were analysed by flow cytometry.

Analytical Determinations in Platelet-Poor Plasma

Fibrinogen (FG) was determined by the Clauss method (ACL TOP 700; Beckman Coulter), FVIII by chromogenic assays and FVII, FIX and FXI by clotting assays (BCS XP system [Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany]). All these substances were determined in PFP samples.

The human urokinase-plasminogen activator (uPA) kit used was from R&D Systems Europe Ltd. (Abingdon, United Kingdom); the tissue plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1) kits were obtained from eBioscience (Hatfield, United Kingdom); and thrombin activatable fibrinolysis inhibitor (TAFI) activity was from American Diagnostica (Pfungstadt, Germany). All these substances were determined in PPP samples, following the manufacturer's instructions and measured in Multiskan FC with incubator (ThermoScientific; Madrid, Spain).

Statistics

The statistical analysis of the experimental data was performed using SPSS 9.0 software (SPSS Inc., Chicago, Illinois, United States). The Shapiro–Wilk test was used to test normal distribution. For comparison of multiple groups, a non-parametric one-way ANOVA using Dunn's multiple comparison post-hoc test was performed. Comparison of the media of two groups was performed using the Student '*t*'-test or Mann–Whitney *U*-test, and the correlations were calculated with Pearson's or Spearman's tests, depending on the sample distribution. All tests were two-tailed and level of statistical significance was set at $p < 0.05$.

Results

Features of Myelodysplastic Syndrome Patients

The mean age of the control group was lower than the mean age of patients with MDS because of the difficulty in obtaining samples from older donors.

To validate our cohort of healthy donors (most of them younger than 65 years), we recruited a group of 16 donors elder than 65 years (most of them accompanying wives or husbands of MDS patients). We analysed by flow cytometry features of their platelets (activation ability, PS exposure) and clot formation kinetics (ROTEM) and compared these results with those obtained in healthy donors younger than 65 years (those recruited in the Blood Donor Section) and we found no significant differences between groups. So, we considered that mean age difference between healthy controls and MDS patients did not bias our results.

Significant differences were observed between controls and MDS patients in red cell counts (controls: $4.2 \pm 0.4 \times 10^6$/µL; MDS patients: $3.1 \pm 0.7 \times 10^6$/µL, $p < 0.0001$), haemoglobin (controls: 13.1 ± 1.4 g/dL; MDS patients: 10.2 ± 2.0, $p < 0.0001$) and monocytes (controls: $6.9 \pm 2.1\%$; MDS patients: $13.8 \pm 2.5\%$, $p < 0.005$), whereas no differences were found in platelet counts (controls: $187 \pm 50 \times 10^3$/µL; MDS patients: 149 ± 710^3/µL, $p = 0.34$) and leukocytes (controls: $5.5 \pm 1.3 \times 10^3$/µL; MDS patients: $5.7 \pm 3.2 \times 10^3$/µL, $p = 0.12$). Twenty-nine percent of MDS patients needed red cells transfusional support.

Global Haemostasis in Patients with Myelodysplastic Syndrome

ROTEM studies were performed with fresh PRP samples to evaluate global haemostasis in MDS patients. ►**Fig. 1** shows a delay in clot formation in the MDS group, as indicated by a prolonged CT (control: 516 ± 93 seconds; MDS: 946 ± 217 seconds, $p < 0.001$), a diminished α angle (control: 62.5 ± 3.3; MDS: 47.4 ± 8.4, $p < 0.0001$) and clot firmness assessed as amplitude at 5 minutes (A5, control: 29.1 ± 3.1; MDS: 23.6 ± 5.1, $p < 0.0001$). This observation was not due to a diminished content of fibrinogen (294 ± 22 mg/dL) or of clotting factors (FVII: $105 \pm 48\%$; FVIII %: 195 ± 21; FIX: $111 \pm 17\%$ and FXII: $102 \pm 21\%$). Finally, samples from MDS patients reached the same MCF as in control group. Surprisingly, a reduced clot lysis after 60 minutes (LY60 control: $88.6 \pm 3.0\%$; MDS: $95.6 \pm 3.1\%$, $p < 0.001$) was observed in MDS samples. This effect was not due to a misbalance in proteins involved in fibrinolysis (►**Table 2**).

To test platelet function in our MDS cohort, platelets were activated with TRAP and binding of PAC1 and exposure of P-selectin was tested. As shown in ►**Fig. 2**, both of them were reduced emphasizing platelet-hampered ability to be activated. Moreover, the worst pathological situation was accompanied by the less ability of fibrinogen receptor to be stimulated.

Apoptosis in Platelets from Myelodysplastic Syndrome Patients

Platelets from patients with MDS presented higher levels of activated caspases-3, -7, -8 and -9 (►**Fig. 3**) and this situation was accompanied by a higher PS platelet surface exposure (►**Fig. 4A**).

Prothrombinase Complex Binding to Quiescent and Activated Platelets

We evaluated if PS was functional for anchoring prothrombinase complex. Binding of FVa and FXa on platelet membrane surface was higher in quiescent platelets from MDS

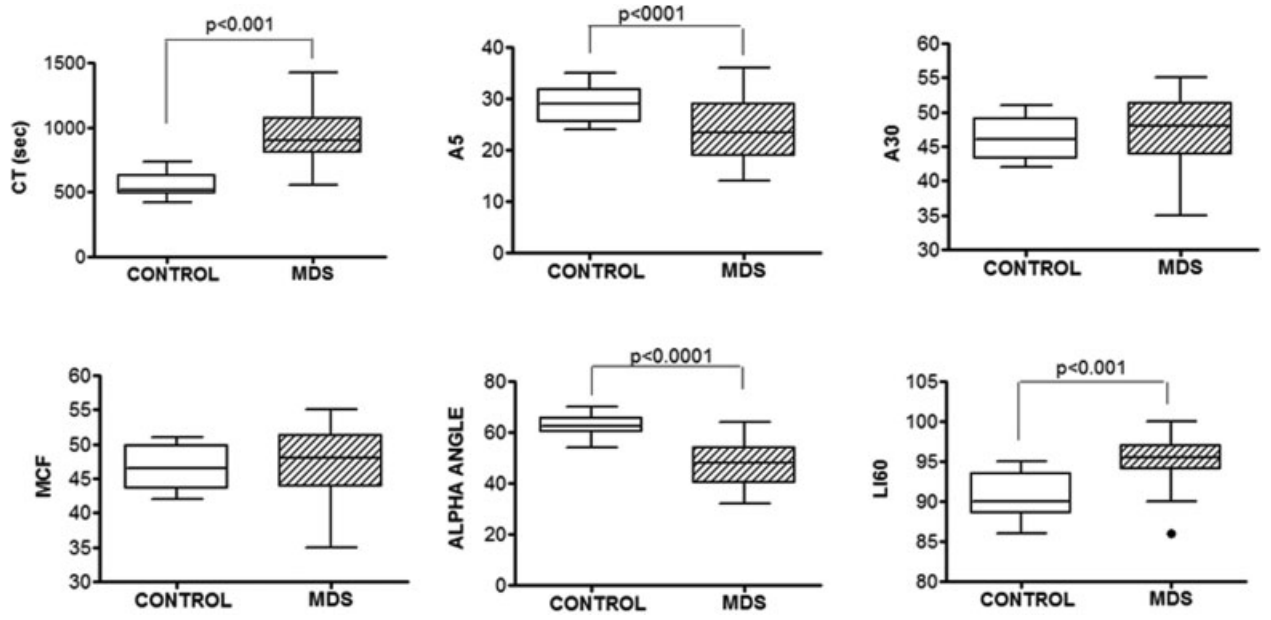

Fig. 1 Features of clot formation in patients with MDS. ROTEM thromboelastography was performed in PRP adjusted to 25×10^3 platelets/μL. Detailed procedures and measured parameters are explained in section 'Methods'. The Mann–Whitney *U*-test was performed and $p < 0.05$ was considered significant. MDS, myelodysplastic syndrome; PRP, plasma-rich protein.

Table 2 Fibrinolysis-related proteins

	Control	MDS
TAFI	36.0 ± 4.9% of activity	29.4 ± 6.4% of activity
uPA	658.2 ± 181 pg/mL	737.5 ± 326.1 pg/mL
tPA	641.0 ± 421 pg/mL	622 ± 329.1 pg/mL
PAI-1	12.9 ± 10.0 ng/mL	11.5 ± 13.2 g/mL

Abbreviations: MDS, myelodysplastic syndrome; PAI-1, plasminogen activator inhibitor-1; TAFI, thrombin activable fibrinolysis inhibitor; tPA, tissue plasminogen activator; uPA, urokinase-plasminogen activator.
Notes: Data are expressed as mean values ± standard deviation. The '*t*' test was performed and none were significant.

patients as shown in ►**Fig. 4B**. After TRAP stimulation, PS exposure increased in both groups, being this increment higher in controls (►**Fig. 4A**). Prothrombinase binding was enhanced after TRAP stimulation and in this situation, no differences were observed between platelets from healthy controls and MDS patients.

Microparticles in Plasma from Patients with Myelodysplastic Syndrome

To determine the cellular source of MPs, flow cytometry analyses were performed and a significant increase was observed in the total amount of MPs due to the increase in monocyte- and red cell-derived MPs in the samples from the patients with MDS (►**Fig. 5**).

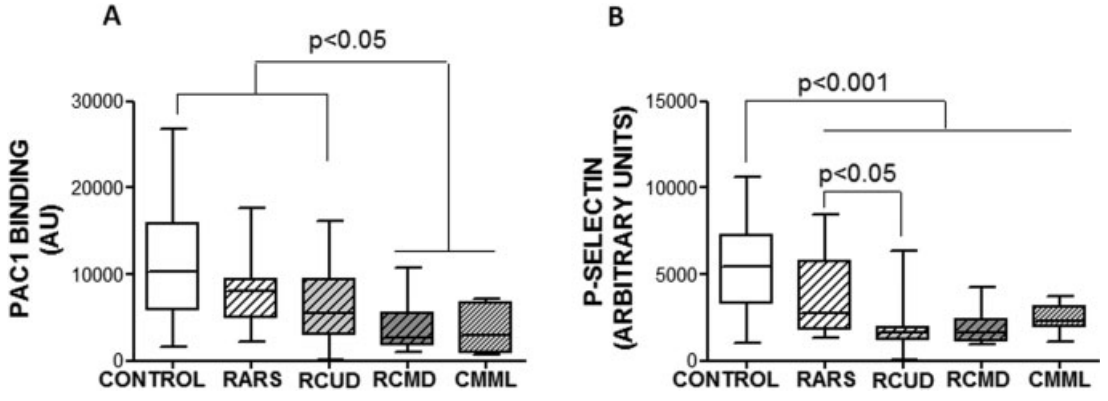

Fig. 2 TRAP-induced activation of fibrinogen receptor and surface expression of P-selectin. Platelets from healthy controls and from MDS patients were stimulated with 100 μM TRAP and then binding of FITC-PAC1 (**A**) and FITC anti-P-selectin mAb (**B**) was assayed and evaluated by flow cytometry. Data are expressed as arbitrary units (mean fluorescence X % of positive cells). Mann–Whitney *U* comparison test was performed and $p < 0.05$ was considered significant. FITC, fluorescein-isothiocyanate; MDS, myelodysplastic syndrome.

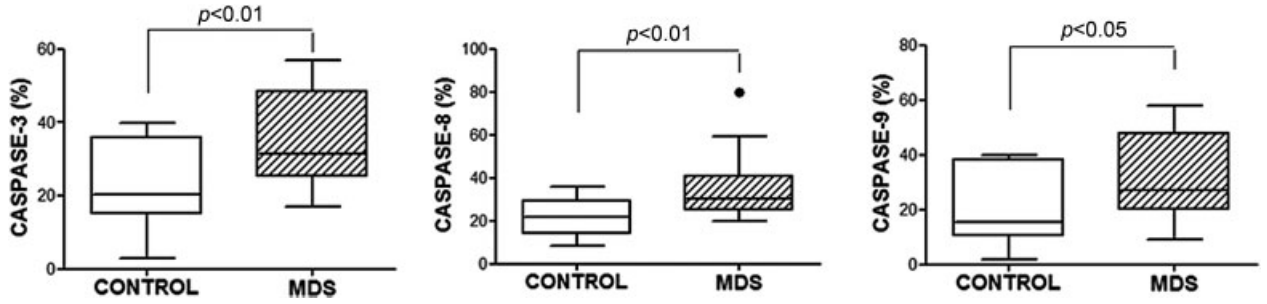

Fig. 3 Platelet-activated caspases -3, -7, -8 and -9 were determined by flow cytometry as described in section 'Methods'. Mann–Whitney *U* comparison test was performed and $p < 0.05$ was considered significant.

Procoagulant activity of MPs associated with either PS or TF was higher in chronic myelomonocytic leukaemia (CMML) patients (►**Fig. 6**).

In the patients with MDS, TF-MP-associated thrombogenic ability correlated with monocyte percentage (Spearman's $r = 0.5019$, $p < 0.01$).

Plasma Coagulant Capacity

Plasma procoagulant activity evaluated by CAT did not show differences between controls and MDS patients in any of the measured parameters: lag time (control: 4.8 ± 3.6 minutes; MDS: 6.3 ± 4 minutes, $p = 0.07$), peak (control: 248.8 ± 59.1 nmol/L; MDS: 227.4 ± 52.1 nmol/L, $p = 0.87$) and ETP (control: 1,455.8 ± 333.0 nmol/L × min; MDS: 1,243.4 ± 256 nmol/L × min, $p = 0.25$).

Since platelets have a key role in blood coagulation, their inclusion in CAT experiments seems to give a more detailed picture of the procoagulant ability than PPP. Considering that a plasma factor might be responsible for the delay in clot formation observed in ROTEM test, the effect of plasma from MDS patients and from controls on the procoagulant capacity of platelets from healthy controls was tested. Under this experimental condition, PPP from the patients with MDS, compared with the PPP from healthy subjects, prolonged lag time for thrombin generation (control: 10.6 ± 2.7 minutes; MDS: 13.3 ± 2.2 minutes, $p < 0.05$), diminished peak (control: 233.7 ± 93.7 nmol/L; MDS: 122.7 ± 54.7 nmol/L, $p < 0.005$) and ETP (control: 1,744.8 ± 376.9 nmol/L × min; MDS: 1,276.4 ± 280 nmol/L × min, $p < 0.001$). All these parameters correlated with ROTEM CT (lag time: Pearson's $r = 0.8833$, $p < 0.001$; peak: Pearson's $r = -0.8300$, $p < 0.001$ and ETP: Pearson's $r = -0.8350$, $p < 0.005$). As previously mentioned, plasma from the patients with MDS possessed normal levels of fibrinogen and clotting factors, so this might not be the cause of the reduced procoagulant profile.

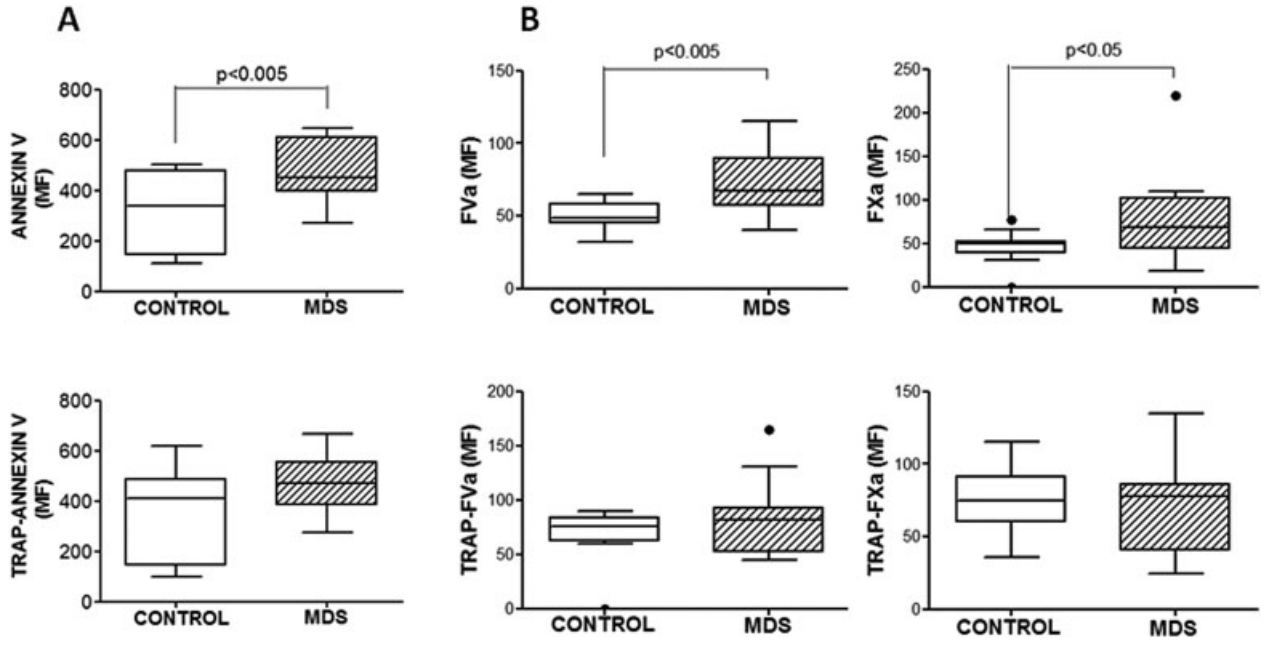

Fig. 4 PS surface exposure and prothrombinase complex binding to platelets from MDS patients. PS surface exposure determined by binding of FITC-Annexin V (**A**) and FVa and FXa binding (**B**) in either quiescent or 100 μM-TRAP stimulated platelets from controls and MDS patients was determined by flow cytometry analysis. Data are expressed as MFps. The Mann–Whitney *U*-test was performed and $p < 0.05$ was considered significant. FITC, fluorescein-isothiocyanate; MDS, myelodysplastic syndrome, MF, mean fluorescence; PS, phosphatidylserine.

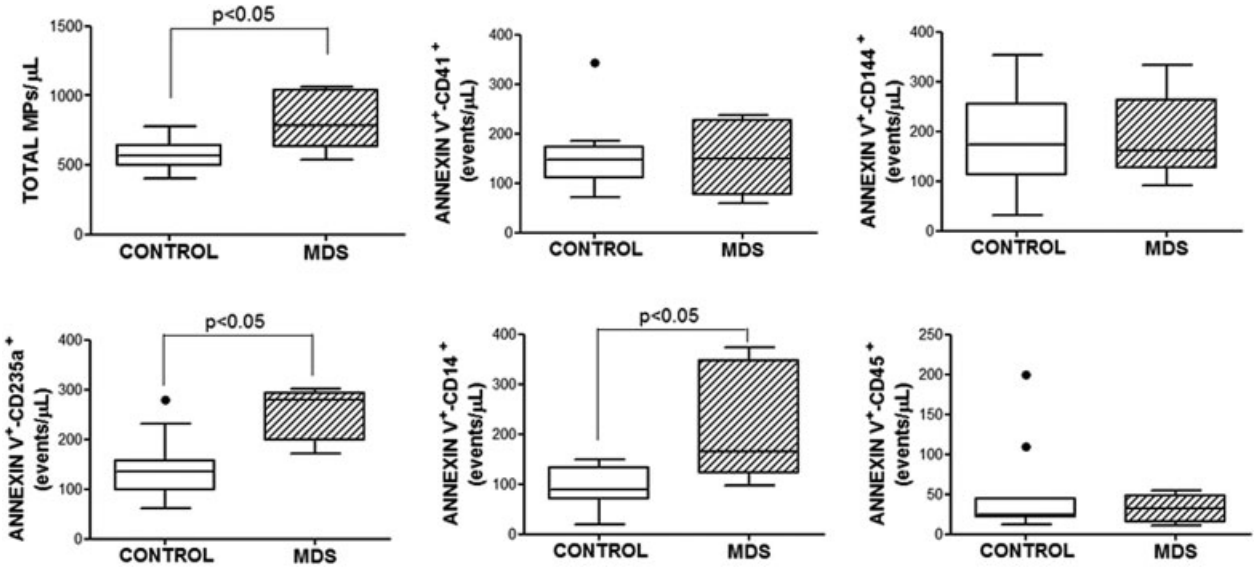

Fig. 5 Analysis of cellular origin of MPs. MPs were labelled with FITC-Annexin V and a PE-labelled antibody specific for a cellular type. Samples were analysed by flow cytometry. The Mann–Whitney *U*-test was performed and $p < 0.05$ was considered significant. FITC, fluorescein-isothiocyanate; MP, microparticle; PE, phycoerythrin.

The effect of plasma from MDS patients on thrombin generation in the presence of control platelets was similar to that observed with plasma from patients with lupus anticoagulant antibodies. Since approximately 45% of patients from our cohort have an autoimmune-inflammatory accompanying condition (AIC), we analysed our results stratifying population in MDS without/with AIC. As observed in ►**Fig. 7**, the presence of AIC seems to hamper thrombin generation in MDS patients.

Discussion

Analyses of the ROTEM parameters of our cohort of MDS patients suggested the existence of at least two antagonistic processes: one of them giving a hypocoagulable pattern (prolonged CT and lower α angle) and another conferring a procoagulant profile (decreased fibrinolysis).

Retarded haemostasis may rely on platelet count and/or function.[14,15] First of these possibilities can be ruled out as experiments were performed in PRP samples adjusted to the same platelet count. Moreover, extra factors that might exist in samples from thrombocytopaenic subjetcs[16] can also be discarded because whole blood from healthy controls and MDS patients had similar platelet count. On the contrary, platelet function seems to be involved because it is hindered (present results and the results of Martín et al[5]). A platelet-reduced ability to be stimulated was also reported by other laboratories.[17,18] In further support of this observation, analyses of platelet's proteome from MDS patients showed a significant reduction in expression of proteins that play critical roles in fibrinogen receptor signaling and thus platelet spreading and aggregation.[19] Diminished capacity of platelets from MDS patients to be stimulated was not an artefact due to the difference in mean age between MDS and healthy control groups since we validated control group and previous works indicated a moderate increase in platelet reactivity with age,[20,21] the opposite of observed in MDS patients.

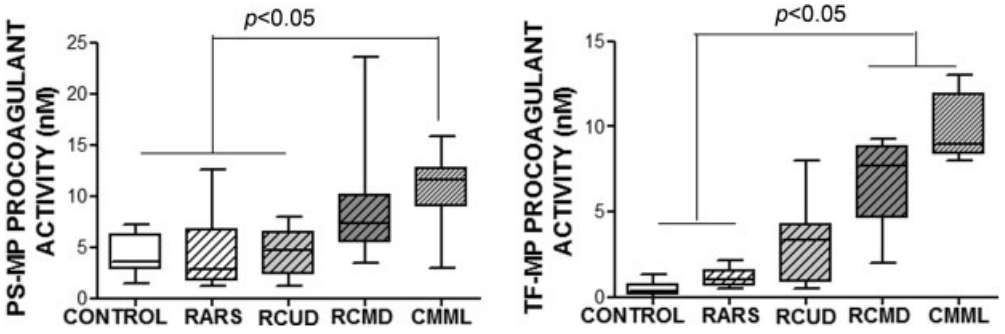

Fig. 6 MP-associated procoagulant capacity in MDS patients. PS- and TF-MP-associated procoagulant activity was determined with ZYMUPHEN MP-Activity kits. One-way ANOVA and Dunn's multiple comparison tests were performed and $p < 0.05$ was considered significant. MDS, myelodysplastic syndrome; MP, microparticle.

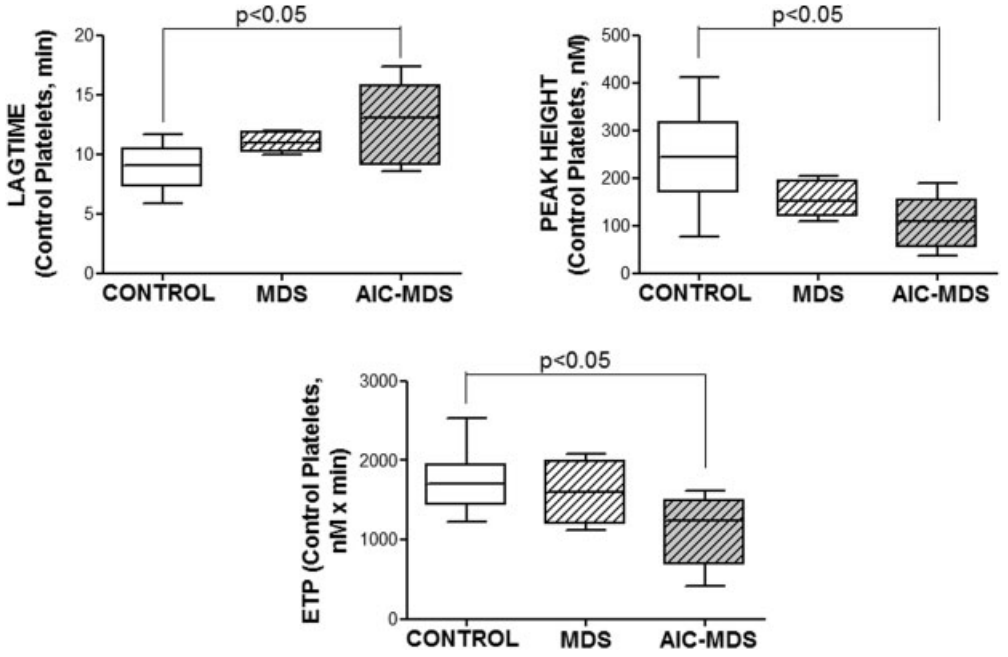

Fig. 7 Effect of plasma from MDS patients on thrombin generation in presence of control platelets. MDS patients were divided into two groups depending on the absence or presence of AICs. Washed control platelets were resuspended and adjusted to 25 $\times$ 10^3 platelets/μL with PPP from controls or patients with MDS or AICs-MDS. Coagulation was triggered by stimulation with 100 μmol/L TRAP. One-way ANOVA and Dunn's multiple comparison tests were performed and $p < 0.05$ was considered significant. MDS, myelodysplastic syndrome.

Impairment of platelet function might be related to increased apoptosis observed in platelets from MDS patients (present results and the results of Martín et al[5]). A similar relationship between platelet apoptosis and function was described for busulfan-treated platelets, which displayed an increased expression of Bax and caspase 3 activity in a dose-dependent manner and presented a reduced collagen- and ADP-induced platelet aggregation.[22]

Platelet's surface membrane almost exclusively exposes choline phospholipids and sphingomyelin, whereas the inner leaflet possesses the majority of the amine-containing phospholipids (PS and phosphatidylethanolamine[23]). Membrane phospholipid asymmetry is disrupted by either activation or apoptosis, despite both processes are mediated by different intracellular mechanisms.[24] In MDS patients, increased PS membrane exposure observed even in quiescent conditions seemed to be related to apoptosis and not activation.[5] PS exposed at the surface of platelets provides a negatively charged scaffold for the binding of calcium-dependent coagulation enzymatic complexes and provides a catalytic surface for the tenase and/or prothrombinase complexes that promote thrombin generation.[25] One of the goals of this work was to demonstrate that MDS platelets were able to bind more prothrombinase complex in basal conditions and a similar amount than platelets from healthy controls after being stimulated. This last observation might be explained because MDS platelets were refractory to being stimulated; so, after TRAP activation, PS exposure was similar in both groups. These procoagulant platelets mount and sustain a procoagulant response; thus, they are proposed to play a significant role in regulating both normal haemostasis and pathological thrombus formation.[26]

Another process triggered by either cellular activation or apoptosis is the release of MPs, exocytic products derived from plasma membrane. The haemostatic and/or thrombotic function(s) of MPs may be related to their surface exposure of PS which allows for docking prothrombinase complex[25] and the presence of specific receptors[27] and cytosolic proteins as well as genetic information in the form of messenger RNA (mRNA) and microRNA from their parental cells.[28] In this study, we observed an increased procoagulant activity associated with PS as well to TF content of MPs. In our cohort of MDS patients, augmented MPs came from red cells and monocytes.

Monocytes, as well as their released MPs, are rich TF containing elements. TF is the transmembrane receptor for factor (F) VII/VIIa and is proposed to be the principal initiator of coagulation in vivo[29] and TF-MPs are considered the main reservoir of plasma TF activity.[30] Monocyte MPs may contribute to confer a procoagulant feature to MDS patients that might protect them for bleeding. It has been reported that fibrin formation is the result of TF activity from MPs and that the accumulation of fibrin in its network formation increases its resistance to fibrinolysis.[31,32] According to our results, TF-MPs increment, and no variations in levels of proteins involved in fibrinolysis, might explain the resistance to fibrinolysis observed in MDS patients. TF-positive MPs were proposed to contribute to thrombosis in a variety of diseases including acute myocardial infarction,[33] sickle cell disease,[34] endotoxaemia[35] and cancer.[36] Moreover, the observation that CMML patients presented the highest procoagulant activity associated with TF-MPs is in accordance with the increased count of monocytes observed in these patients.[37]

Increased MPs from red cells might also favour a procoagulant profile in MDS patients since they initiate thrombin generation via the intrinsic pathway of coagulation, apparently, by a mechanism completely independent of TF.[38] In support of this, the hypercoagulable state in patients suffering from sickle cell disease is associated with increased levels of circulating red cell MPs,[39] and, consistently, haemolysates containing red cell MPs, augmented thrombin generation.[40]

Despite we did not find differences in platelet-derived MPs between MDS patients and controls, when results were related to the platelet count, subjects with MDS had higher platelet-derived MPs/platelets ratio. Similar results were reported by Connor et al[41] reflecting an increased number of MPs released by each platelet.

It is not uncommon for patients with MDS to present autoimmune and inflammatory conditions (AICs) that can appear before, during or after this disease diagnosis.[42] Constitutively activated innate immune and inflammatory pathways may be responsible of AICs. AICs could arise from cellular stresses associated with immunologic senescence of the aged MDS population or to genomic instability and other genetic and epigenetic abnormalities that occur in haematopoietic cells due to abnormal cellular interactions in the BM microenvironment.[43,44]

We observed that nearly the 40% of the MDS patients included in our cohort presented immunological (anti-neutrophil, anti-nuclear and anti-*Saccharomyces cerevisiae* antibodies) or/and serological (increased rheumatoid factor and C-reactive protein) abnormalities or an autoimmune disease. Similar immunological abnormalities were reported in studies performed on Asian[45] and European MDS populations.[46]

Whether the hampered effect of plasma on control platelets in the thrombin generation experiments may be related to antibodies against platelets is an issue that deserves more studies. In support of the possibility of the existence of this kind of antibodies, it has been reported that some MDS patients with thrombocytopaenia had a recovery of their platelet count after splenectomy.[47]

Our work shows that haemostasis in MDS patients is a complex process influenced by multiple factors affected by disease etiopathogenesis, comorbidities accompanying elderly patients. Moreover, many drugs used for MDS treatment modify platelet count. Azacitidine doubles platelet count in patients with thrombocytopaenia and with normal platelet in all cytogenetic risk groups, in patients with and without circulating blasts, and in patients who were transfusion dependent and independent.[48] Decitabine shows an increased platelet count within two cycles in most MDS patients,[49] whereas lenalidomide's main side effect is thrombocytopaenia grade 3 or grade 4.[50] We recommend a close follow-up of haemostatic state in MDS patients before and during drug therapy to avoid either haemorrhagic or thrombotic complications. Thromboelastography with the ROTEM system maybe a valuable tool for studying haemostasis because it provides real-time analysis of the viscoelastic properties of clot formation and dissolution.

What is known about this topic?

- MDS patients, even those with normal platelet count, have a reduced platelet function due to enhanced apoptosis but do not bleed in many cases, suggesting the presence of additional mechanisms different from platelet count/function that participate in controlling their haemostasis.
- Several drugs used for MDS treatment modify platelet count, with probable effects on haemostasis.

What does this paper add?

- This work evaluated global haemostasis in MDS patients showing the existence of two antagonistic processes: one of them gives a hypocoagulable pattern and another confers a procoagulant profile.
- Hypocoagulability might rely on impaired capacity of platelets from MDS to be activated.
- Procoagulant profile seemed to be related to a decreased fibrinolysis and to the increase in tissue factor-containing microparticles and in the binding of prothrombinase complex to platelets which, due to the enhanced apoptosis, exposed more phosphatidylserine.
- Evaluation of haemostasis during therapeutic treatments of MDS arises as a recommended tool to avoid the occurrence of adverse haemostatic events.

Note
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Authors' Contribution
I.F.B., R.J.S., E.M.M. and E.G.A-S. performed the experiments. R.d.P., M.T.A.R., M.M.S., M.I.R.P. and V.J-Y. diagnosed and provided clinical care to MDS patients. I.G. collected and verified patient information. I.F.B., R.d.P., V. J-Y. and N.V.B. analysed the results. N.V.B. was the principal investigator, designed the experiments and wrote the manuscript. All authors approved the manuscript.

Conflict of Interest
None.

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